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Protectiveness, and Mode of Action

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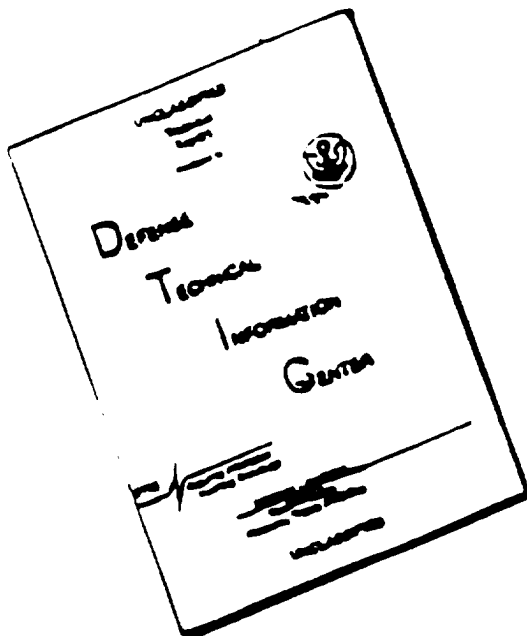
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J. C. Schady 9/17/96  
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**Midterm Report for Collaborative Agreement DAMD17-94-C-4041**  
**Year 1: September 1, 1994-August 31, 1995**  
**Year 2: September 1, 1995-August 31, 1996**

**Introduction**

This project is evaluating the *Yersinia pestis* (plague) virulence protein YopM as a prospective component of a subunit plague vaccine. YopM is one of a set of proteins that are secreted into the culture medium by *Y. pestis* under conditions that are believed to mimic the contact of the bacteria with a phagocytic cell (1). Two of these proteins are directly targeted from the bacterium into the phagocyte, with resulting paralysis of phagocytosis. The fate of YopM is not known and will be investigated in this project (Aim 3). It is thought that at least some of YopM is likely to be present in the extracellular surroundings in tissues, because we have shown that YopM binds human  $\alpha$ -thrombin sufficiently tightly to prevent activation of platelets (2,3), and  $\alpha$ -thrombin is generated extracellularly by the activation of the serum protein prothrombin (4). YopM does not bind prothrombin (3); hence YopM is believed to act by sequestering thrombin as it is generated from prothrombin at foci of infection (1,3). This would be expected to have an anti-inflammatory effect, which could contribute to the virulence of *Y. pestis*, and indeed we found that YopM is necessary for the full virulence of *Y. pestis* in mice (2). Because YopM is thought to be located extracellularly, where it would be accessible to antibody, and is necessary for full virulence of *Y. pestis*, its activity might be neutralizable by antibody, thus affording some protection against plague. This is the basic hypothesis that is being tested in the Collaborative Agreement DAMD17-94-C-4041.

The three Aims in the Collaborative Agreement will examine different aspects of the hypothesis. The studies of Aim 1 will generate reagents (pure YopM and antibody against YopM) that will be used in all aims and will use these reagents to characterize the immune response to YopM and to test whether mice that are actively or passively immunized against YopM are protected against plague. These studies will directly ask the question of whether YopM is a protective antigen. Aim 2 will determine if thrombin-binding is the main function of YopM during an infection. We will make a *Y. pestis* KIM strain that expresses a mutant YopM that is defective in binding thrombin. If this *Y. pestis* strain is as avirulent as a strain that expresses no YopM at all, then thrombin-binding is YopM's main function; if not, then the existence of additional activities of YopM will have been revealed. In Aim 3, we will test the assumption that YopM is extracellular by determining whether YopM is secreted to the medium or into phagocytes or both when *Y. pestis* contacts a macrophage. If all of YopM is secreted into the medium, then the extracellular nature of YopM will be supported. If some or all of YopM enters macrophages, then we will have learned that YopM likely has a function other than, or in addition to, thrombin-binding. The studies of Aims 2 and 3 will better characterize the mechanism of action of YopM, and the findings may be useful when considering



potential toxicity of YopM to vaccinees.

## Body

### Aim 1. Assess the immunogenicity and protective capacity of YopM

Technical objective (TO) 1. Determine if anti-YopM antibodies provide significant protection against lethal *Y. pestis* challenge.

This TO was accomplished during this year's work. The Year 1 annual report (a copy is attached as an appendix) described the purification of YopM to be used to immunize mice and the preparation of mouse  $\alpha$ -YopM antibody to be used for passive immunization (although the latter was still underway at the time of the report). We provided pure YopM to Col. Friedlander's plague research group at USAMRIID in fulfillment of the YopM deliverable. We recently sent purified mouse anti-YopM to fulfill the  $\alpha$ -YopM deliverable of this Collaborative Agreement. What remained in this TO was to test for the ability of antibody to YopM to protect mice against challenge by *Y. pestis* KIM5.

Removal of endotoxin from YopM, a final test. One aspect of reagent-generation that carried over into year 2 of this project was our interest in finding a less expensive method of removing endotoxin from preparations of YopM to be injected into mice. We purchased a new product, Acticlean ETOX resin (Sterogene Bioseparations, Inc., Carlsbad, CA) and followed their protocol, including their suggestions for how to treat acidic proteins. None of these tests was successful, although the product did successfully remove endotoxin from preparations of histidine-tagged LcrV used as a control protein. The resin retained most of the YopM loaded onto it. Specifically, we tried the following, using a 5 ml column.

1. 1 ml of 2 mg/ml YopM in 0.05 M Na acetate pH 5 was run through a column equilibrated in this buffer. Result: both YopM and endotoxin were retained on the column.
2. We tried dissolving YopM in 0.05 M Na acetate pH 4.5 to run through a column equilibrated at that pH, as suggested by the Sterogene technical representative, but YopM would not go into solution.
3. We tried loading YopM (2 mg/ml) in equal volume to that of the column (5 ml), also suggested by Sterogene. YopM was still lost on the column as in 1.
4. We tried including 1% deoxycholate in the 0.05 M Na acetate pH 5 buffer: a white precipitate formed while equilibrating the column, and this test was discontinued.
5. The column was equilibrated with 0.1% Na deoxycholate in PF-PBS, and 5 ml of 2 mg/ml YopM in PF-PBS was run through the column. YopM again was lost.
6. The column was equilibrated with 0.25 M Na acetate, and ca. 5

ml 2 mg/ml YopM in this buffer was run through. YopM was still retained on the column.

We did not make any further attempts to find novel methods of removing endotoxin from YopM and treated our YopM as needed with End-X B52 resin (Associates of Cape Cod, Inc., Woods Hole, MA), previously shown to be effective (but expensive).

Passive protection studies. We made passive protection tests using both mouse  $\alpha$ -YopM and, because this reagent was limited in amount (see the Year 1 annual report), we provided rabbit  $\alpha$ -YopM as well as a positive control rabbit antibody against LcrV ( $\alpha$ -HTV) to this project for protection tests. We had not requested use of rabbits in this Collaborative Agreement, but we had appropriate antibodies on hand, as we had produced them for a concurrently running NIH-supported project in our lab. These rabbit and mouse reagents are briefly described below.

Antibody reagents produced in rabbits. Two New Zealand White rabbits were immunized subcutaneously (SC) as previously described (5) with 100  $\mu$ g of purified YopM in PBS (pH 7.4) emulsified 1:1 with Freund's adjuvant (FA) (Difco Laboratories, Detroit, MI) and boosted bimonthly SC with 100  $\mu$ g of YopM in PBS and FA. In this and all other uses of FA, the first dose contained the complete adjuvant, while subsequent doses contained incomplete adjuvant (Difco).  $\alpha$ -YopM serum antibody titers were monitored by ELISA, and the specificity of the antiserum was determined by Western blot of *Y. pestis* cell extracts (data not shown). Anti-HTV antibodies were produced similarly, using a full-length *Y. pestis* V antigen fused to a 19-residue leader peptide containing 6 histidine residues (HTV), raised in *Escherichia coli* and purified on a Ni-NTA column according to the manufacturer's protocol (Qiagen, Inc., Chatsworth, CA). IgG from rabbit antisera was affinity purified by chromatography on protein A sepharose CL-4B (Sigma Chemical Co., St. Louis, MO) or by Affi-Prep protein A column (BioRad Laboratories, Hercules, CA) according to the manufacturer's recommended protocol.

Mouse anti-YopM and anti-BSA serum and ascites. Female BALB/c mice ages 12-13 weeks were bled (day 0) and intraperitoneally (IP) given 40  $\mu$ g of YopM in pyrogen-free phosphate buffered saline (PF-PBS) + FA or only PF-PBS + FA on days 2, 16 and 37. Seven to 14 days later, they were injected IP with 300  $\mu$ l of Pristane (2,6,10,14-tetramethylpentadecane, Sigma). Approximately one week later, mice were injected IP with  $1.2 \times 10^6$  XS63 cells, a non-secreting mouse myeloma cell line (American Type Culture Collection, Rockville, MD) in Hank's Balanced Salt Solution (Gibco BRL, Grand Island, NY). Ascites developed within three weeks post injection of the myeloma cells and was collected as it formed using a 20 G needle and a 10 cc syringe. In the process of raising mouse anti-YopM antibody, we used these mice to compare the efficacy of two adjuvants, FA and Adjuvax (AV) (Alpha Beta Technology, Worcester, MA). AV was added as a powder to YopM in PBS and mixed by multiple draws through an 18 G needle. 5 mice received YopM:AV and 5 received PBS:AV. After these YopM-immunized

groups of mice reached peak anti-YopM titers, the AV-treated mice were primed with Pristane and subsequently handled as for FA-treated mice. Ascitic fluid was centrifuged to remove cells and debris.

Additional mice were immunized twice, 2 weeks apart, with 40 µg of bovine serum albumin (BSA) (Fraction V, Sigma) or 40 µg YopM in PBS + FA or with PBS + FA alone. These mice were bled biweekly from the tail vein (ca. 0.5 ml blood per sample), and the antiserum was later combined with the ascites, as it proved to have a 10-fold higher concentration of anti-YopM antibody than did ascites.

IgG from the ascites and sera was semipurified by affinity chromatography on Affi-Prep (BioRad) protein A columns following the manufacturer's suggested protocol.

Enzyme-linked immunosorbent assay (ELISA) for  $\alpha$ -YopM,  $\alpha$ -HTV, and  $\alpha$ -BSA antibody levels in sera. This assay was developed during the first year of this contract but is described briefly here, as we applied it for measuring titers of antibodies to BSA and HTV as well as YopM. Flat-bottom, 96-well, Nunc Maxisorp immunoplates (Fisher Scientific) were coated with 100 µl of the desired protein solution (4 µg/ml in PBS) at room temperature (RT) for 2 h (or overnight at 4°C). The wells were blocked with skim milk (200 µl of a 2% [wt/vol] solution in PBS per well, 2 h at RT or 4°C overnight) and washed thrice, 3 min each time, with PBS containing 0.05% (vol/vol) Tween 20 (Fisher) and 0.02% (wt/vol) sodium azide (Sigma) (PBS-TA). Test sera or ascites (primary antibody source) were serially diluted in PBS-TA, and 100 µl of each dilution was added to duplicate wells for 2 h at RT. Control wells were given 100 µl of PBS-TA only. The plates were then washed and coated 2 h at RT with alkaline phosphatase-labeled secondary antibody: goat anti-mouse IgG  $\gamma$ -chain diluted 1:20,000, goat anti-rabbit IgG diluted 1:1500, or, for tests described below with human plague convalescent sera, goat anti-human IgG  $\gamma$ -chain diluted 1:20,000 (Sigma). The wells were washed, 100 µl of Sigma Fast substrate (p-nitrophenyl phosphate) was added to each well, the plates were incubated at RT for 15 min, and the reaction was stopped by the addition of 25 µl of 3N NaOH to each well.  $A_{405\text{ nm}}$  was measured using a v Max kinetic microplate reader (Molecular Devices Corp., Menlo Park, Calif.). Average absorbance values were determined for each dilution of each serum sample, and the antibody titer of each sample was calculated to be the highest dilution at which the absorbance value was greater than 2 times the standard deviation from the average absorbance of control wells.

Passive immunization of mice followed by challenge with *Y. pestis* KIM5. To determine if antibody alone could mediate protection against *Y. pestis* challenge, IgG from mice immunized with YopM or BSA was passively transferred to naive inbred animals. Female BALB/c mice (6-8 wk) were passively immunized IP with a single dose of 500 µg of mouse  $\alpha$ -YopM IgG or negative control ( $\alpha$ -BSA) IgG in 500 µl of PF-PBS. Control mice received 500 µl of PF-PBS, IP. Twenty-four hours after passive immunization, the mice were bled, and pooled serum samples tested by ELISA to determine that  $\alpha$ -YopM or  $\alpha$ -BSA antibodies had entered the serum. Forty-eight hours after immunization, groups of 10 mice were challenged IV via the retro-orbital sinus with decimally increasing doses of *Y. pestis* KIM5 ( $10^1$  to  $10^4$  CFU) in 100 µl of PF-

PBS. In these and all other challenge experiments, the actual CFU given were confirmed by plating. The mice were observed for 17 days after challenge, and the average doses required to kill 50% of the mice ( $LD_{50}$ ) for the treatment groups were calculated (6). Following challenge with *Y. pestis* KIM5, there were no significant differences in CFU of *Y. pestis* required to kill 50% of the animals in any of the three groups, indicating that YopM antibody on its own is not protective (Table 1). However, there appeared to be a slight increase in the  $LD_{50}$  values for animals immunized with  $\alpha$ -BSA or  $\alpha$ -YopM antibody, indicating a small non-specific protective effect of antibody per se (Table 1).

Outbred Swiss Webster mice were also examined to determine if immunization with  $\alpha$ -YopM was protective. Female Swiss-Webster mice were immunized as above with  $\alpha$ -YopM or positive control ( $\alpha$ -HTV) Ig in PF-PBS, while control mice were given PF-PBS. Twenty-four hours later, they were assessed for  $\alpha$ -YopM and  $\alpha$ -HTV antibody as above. Forty-eight hours after immunization, groups of 5 mice were challenged IV with  $10^1$  to  $10^5$  CFU of *Y. pestis* KIM5, observed for 14 days, and  $LD_{50}$  values were determined. However, the presence of  $\alpha$ -YopM antibody also did not increase the  $LD_{50}$  value in these passively immunized mice when compared to control animals (Table 1). Interestingly, 60% of mice given  $\alpha$ -HTV antibody were protected at a challenge dose of  $10^4$ , while all mice at this same dose in the two other treatment groups had died by day 7 post-challenge (data not shown), indicating that antibody against LcrV is protective as previously shown (8) and showing that mice can be protected against experimental plague by our immunization protocol.

These data show that YopM is not protective by passive immunization and will be discussed further below along with the results of active immunization tests.

**Table 1.** Passive immunization of BALB/c and Swiss Webster mice followed by challenge with *Y. pestis* KIM5.

Mouse Strain	Immunogen	µg IgG per mouse	YopM Serum Antibody Titer	LcrV Serum Antibody Titer	LD <sub>50</sub> value (CFU/mouse)
BALB/c	PBS	0	$\leq 10^1$	ND <sup>a</sup>	$4.5 \times 10^1$
	anti-BSA <sup>b</sup> IgG	500	$\leq 10^1$	ND	$2.6 \times 10^2$
	anti-YopM <sup>b</sup> IgG	500	$10^4$	ND	$3.0 \times 10^2$
Swiss-Webster	PBS	0	$\leq 10^1$	$\leq 10^1$	$1.5 \times 10^1$
	anti-HTV <sup>c</sup> IgG	500	$\leq 10^1$	$10^4$	$> 1.0 \times 10^4$
	anti-YopM <sup>c</sup> IgG	500	$10^4$	$\leq 10^1$	$< 1.0 \times 10^1$

<sup>a</sup> ND - not determined

<sup>b</sup> mouse Ig

<sup>c</sup> rabbit Ig

## TO 2. Characterize the immune response in mice immunized with YopM

This TO has two parts: characterize the humoral response to immunization with YopM and characterize the cellular response to immunization with YopM. We completed the first of these during year 1. We will complete the second during the third year of this collaborative agreement. As we found that soluble YopM is a potent immunogen, we would expect that a CD4<sup>+</sup> T helper type 2 response would be found in tests characterizing the cellular response to YopM given as a soluble antigen. As part of characterizing the lymphocyte response to YopM and its potential protective efficacy, we will determine if YopM can elicit a CD8<sup>+</sup> T cell response when provided to mice during a *Y. pestis* infection. If so, we will have in-vivo evidence for an intracellular fate of YopM during an infection, and it would be relevant to pursue this result in future studies to determine if this kind of immune response to YopM is protective and a useful adjunct to a human plague vaccine.

YopM-reactivity in convalescent plague sera. This past year we did one additional experiment that falls under the classification of characterization of the humoral response to YopM. By way of relating our study more directly to its potential relevance for humans, we

determined if three samples of human plague convalescent serum, known by a standard passive hemagglutination test to have specific reactivity for the capsular protein F1, would also have reactivity for YopM and LcrV. Many years ago, we had obtained three samples of human convalescent plague serum from T. Quan (Centers for Disease Control and Prevention, Ft. Collins, CO). Their sample designations and passive hemagglutination (pHA) titers (9, 10) against *Y. pestis* capsular antigen (fraction 1 antigen) were: TX84-220, pHA 1:32; 80NM 912, pHA 1:512; 77NM 697C, pHA 1:32. These sera were tested for YopM and LcrV serum antibody titers using the indirect ELISA method described earlier.

All of the samples had reactivity against YopM (titers 1:800-1:1600 by ELISA) and LcrV (titers 1:1600 to  $\geq$ 1:3200 by ELISA). The serum sample with the highest agglutination titer to F1 (1:512) also had the highest Ab titer to LcrV ( $\geq$  1:3200 [highest dilution tested]) but had a lower titer to YopM (1:800). In contrast, the serum sample with the highest titer to YopM (1:1600) had a relatively lower titer to LcrV (1:1600) and a low agglutination titer to F1 (1:32). Accordingly, as with the known protective antigens F1 and V antigen, YopM also is expressed sufficiently during human plague to elicit a specific immune response. We speculate that YopM is sufficiently present in infection of humans to exert its virulence effects.

TO 3. Determine if an active immune response to YopM protects mice against lethal *Y. pestis* challenge. We had not done any of this work in year 1 but completed this TO in year 2. We tested the protective efficacy of active immunization, as this protocol might prime effector cells which may be required in addition to YopM-specific antibody to protect against *Y. pestis* challenge.

Purification of histidine-tagged LcrV. We used HTV as a positive control, protective antigen in some tests and prepared this antigen as follows. *Escherichia coli* DH5  $\alpha$  (Gibco BRL) containing pHTV carrying the *Y. pestis* lcrV sequence cloned into the pPROEX-1 expression vector (Gibco BRL) had been constructed in the course of our NIH-funded concurrent project. It was grown in 200 ml of LB broth (11) containing ampicillin (100  $\mu$ g/ml) at 37°C to an  $A_{620nm}$  of 0.8. Production of HTV was induced with 0.5mM isopropylthio- $\beta$ -D-galactoside (IPTG) (Life Technologies, Grand Island, NY) followed by a further 3 h incubation at 37°C. The cells were pelleted, resuspended in nickel-NTA column Buffer A (protocol of Qiagen Inc.), and lysed by explosive decompression in a French press, after which insoluble fragments and unlysed cells were removed by ultracentrifugation. HTV was purified from the supernatant using a 1 ml nickel-NTA column and following the manufacturer's suggested protocol. Its purity was estimated by SDS-PAGE on a 12.5% (wt/vol) gel followed by silver staining. Endotoxin concentrations were confirmed by limulus amoebocyte lysate (LAL) assay (BioWhittaker Inc., Walkersville, MD) to be  $\leq$  1ng per 40  $\mu$ g of protein.

Active immunization of mice followed by challenge with *Y. pestis* KIM5. In year 1 as part of TO 2, we had determined the optimum adjuvant (FA), route of administration of antigen (IP), timecourse of the antibody response (maximal by 15 d), and character of the antibody

response to YopM (i.e., amounts of  $\alpha$ -YopM isotypes and subclasses: predominantly IgG1) for active immunization of mice with pure YopM.

For challenge with *Y. pestis*, female, 8-week old BALB/c mice were immunized IP biweekly for 6 weeks with 0.2 ml containing 40  $\mu$ g of YopM in PF-PBS emulsified 1:1 with FA or PF-PBS + FA alone (control mice). Unless otherwise specified, titers of relevant antibodies (here,  $\alpha$ -YopM) were assessed in active immunization experiments by ELISA 24 h before each immunization and 1 d before challenge. Eighteen days after the third and final boost, groups of 5 mice were challenged IV with decimally increasing doses ( $10^1$  to  $10^7$  CFU) of *Y. pestis* KIM5 in 100  $\mu$ l of PF-PBS. The mice were observed for 24 days post-challenge, and LD<sub>50</sub> values were determined. In this first study, the  $\alpha$ -YopM antibody titer in pooled serum from immunized BALB/c mice was  $10^5$  by ELISA at the time of challenge. In PBS-treated mice the  $\alpha$ -YopM antibody titer was  $<10^2$  (lowest dilution tested) in pooled sera. In both treatment groups mice began to die 2 days post-challenge with the highest challenge dose. The LD<sub>50</sub> in the YopM immunized group of mice was not significantly different from that of the PBS-treated group following challenge with *Y. pestis* KIM5 (Table 2), indicating that YopM is not a protective antigen.

We were concerned that the high dose of YopM given for immunization might not have been completely eliminated by the mice by 18 d after the final boost (at time of challenge) and might have had a residual effect on host defenses at challenge. Accordingly, we modified the immunization protocol to give a smaller amount of YopM immunogen with only one boost and a longer time period (1 month) between the last immunization and challenge. Specifically, 6-8 week-old female BALB/c mice were immunized biweekly twice with a reduced amount of YopM (20 $\mu$ g) in PF-PBS + FA. A second, positive control group of mice was immunized with 20  $\mu$ g HTV following the same regimen. Negative control mice received PF-PBS + FA or PF-PBS alone. Groups of 10 mice in each of the treatment categories were challenged IV with  $10^1$  to  $10^7$  CFU of *Y. pestis* KIM5 one month after the second immunization. The mice were observed for 24 days post-challenge, and LD<sub>50</sub> values were determined. As with the first study, the  $\alpha$ -YopM titers were  $\geq 10^5$  in pooled immune mouse serum and  $< 10^1$  in control mouse serum. Upon challenge there was no significant difference in the LD<sub>50</sub> value when immunized and control mice were compared (Table 2), again supporting the finding that active immunization with YopM is not protective. Freund's adjuvant had a slight non-specific protective effect (Table 2).

Because BALB/c mice as a group possess only one allotype (H-2<sup>d</sup>) of the major histocompatibility (MHC class II) locus required for antigen presentation (7), they are limited in their repertoire of presentable peptides and may not effectively present YopM peptide fragments to immune effector cells. Therefore, we immunized and challenged outbred mice (Swiss-Webster), which as a group would possess a spectrum of MHC loci and thus be able to present a wider variety of peptides to effector cells. Female 6-8 week-old Swiss-Webster mice were immunized IP biweekly twice with 20  $\mu$ g of YopM + FA or HTV + FA; negative control mice were given PF-PBS + FA. Serum  $\alpha$ -YopM and  $\alpha$ -HTV titers were quantitated two weeks after the second

immunization. One month after the second immunization, groups of 5 mice were challenged IV with  $10^1$  to  $10^5$  CFU of *Y. pestis* KIM5. The mice were observed for 17 days post-challenge, and LD<sub>50</sub> values were determined. Before challenge, YopM-immunized mice collectively had a specific serum antibody titer of  $10^5$  while control mice had  $\alpha$ -YopM titers of  $< 10^1$ . HTV-immunized animals had a pooled  $\alpha$ -HTV serum antibody titer of  $> 10^5$ , while pooled serum samples from the two other groups groups of mice had titers  $< 10^1$ . Similar to the findings of the first experiment, active immunization with YopM did not significantly increase the LD<sub>50</sub>

**Table 2. Active immunization of BALB/c and Swiss Webster mice followed by challenge with *Y. pestis* KIM5.**

Mouse Strain	Expt. No.	Immunogen	$\mu$ g immunogen /mouse <sup>a</sup>	YopM Serum Antibody Titer	LcrV Serum Antibody Titer	LD <sub>50</sub> value (CFU/mouse)
BALB/c	1	PBS + FA	n.a.	$\leq 10^1$	n.a.	$4.5 \times 10^3$ <sup>b</sup>
		YopM + FA	40	$10^5$	n.a.	$1.4 \times 10^3$ <sup>b</sup>
	2	PBS	n.a.	$\leq 10^1$	n.a.	$< 1.0 \times 10^1$
		PBS + FA	n.a.	$\leq 10^1$	n.a.	$1.0 \times 10^1$
		YopM + FA	20	$10^5$	n.a.	$1.2 \times 10^1$
Swiss-Webster	1	PBS + FA	n.a.	$\leq 10^1$	$\leq 10^1$	$< 1.0 \times 10^1$
		YopM + FA	20	$10^5$	$\leq 10^1$	$3.0 \times 10^1$
		HTV + FA	20	$\leq 10^1$	$\geq 10^5$	$> 1.0 \times 10^5$

<sup>a</sup> n.a. - not applicable

<sup>b</sup> The LD<sub>50</sub> values for this experiment were larger than for others with BALB/c mice, probably because the mice were older at the start of the experiment and then went through a more prolonged immunization than in other experiments.

values for these mice over those for controls (Table 2). Mice immunized with HTV demonstrated a significant degree of resistance to challenge, as all mice survived even the highest challenge dose, indicating, as previously shown (12,13), that LcrV is a protective antigen.

Effect of exogenous YopM on *Y. pestis* and *L. monocytogenes* challenge in outbred mice. Because it was conceivable that YopM could have an extracellular anti-host role without being neutralizable by antibody, we tested whether exogenously supplied



YopM exacerbated infections by homologous (*Yersinia*) and heterologous (*Listeria*) pathogens. Because only a few *Y. pestis* KIM5 bacteria kill mice (tables 1 and 2), we would not expect to measure reliably an exacerbating effect of YopM treatment on infection by this strain. Accordingly, we used the YopM<sup>-</sup> *Y. pestis* KIM5-3233, previously shown to be attenuated in BALB/c mice (2) and tested for the ability of exogenous YopM to reconstitute virulence.

On day 1, groups of 10 female 5-6 week Swiss Webster mice were challenged IV via the retro-orbital sinus with  $10^1$  to  $10^4$  CFU of *Y. pestis* KIM5-3233, or  $10^1$  to  $10^4$  CFU of *L. monocytogenes* EGD in 100  $\mu$ l of PF-PBS. Approximately 2 h later, 5 mice for each challenge dose were administered PF-PBS and the other 5 mice received 100  $\mu$ g YopM in 100  $\mu$ l of PF-PBS, given in the other eye. On d 2 the YopM-supplemented mice received YopM IV while on days 3, 4, 5, and 6 post-infection, they received IP injections of 100  $\mu$ g of YopM in 100  $\mu$ l of PF-PBS. This test was made twice with similar results, once using YopM not cleaned of endotoxin; the data shown in Table 3 are for endotoxin-free YopM.

To assess the effect of antibody on the action of exogenous YopM, 40 mice were injected IP with 500  $\mu$ g of rabbit  $\alpha$ -YopM antibody or irrelevant rabbit antibody (from pre-immune serum) on days 0 and 2. On day 0, ca. 4 h after receiving the antibody, groups of 10 mice were challenged retro-orbitally with  $10^1$  to  $10^4$  CFU of *Y. pestis* KIM5-3233 and then were given YopM or PBS in the other eye as above. On day 2, both YopM and antibody were administered IP. To minimize interaction of YopM and antibody in the peritoneum these proteins were administered 6h apart. Additional groups of 5 control mice were given either PF-PBS or YopM but were not challenged. The LD<sub>50</sub> and MTD values for each treatment group was determined.

YopM treatment did not exacerbate a *Listeria* infection (Table 3). However, YopM did enhance the virulence of by the YopM<sup>-</sup> *Y. pestis*, causing a more than 3-fold decrease in the LD<sub>50</sub>. This effect was swamped out by the nonspecific protective effect of large doses of any antibody (also see Table 1), so we were not able to determine whether anti-YopM antibody can neutralize exogenous YopM's virulence-enhancing effect. These findings indicate that YopM does not act by itself to counteract host defenses important for resistance to *Listeria* but that exogenously supplied YopM might have a virulence-promoting effect on *Yersinia*. This raises the possibility that YopM might have an extracellular function.

Table 3. Effect of exogenous YopM on virulence of *Y. pestis* and *L. monocytogenes*.

Bacterium	Exogenous YopM <sup>a</sup>	Antibody <sup>b</sup>	MTD <sup>c</sup>	LD <sub>50</sub>
<i>Y. pestis</i> KIM5-3233 <sup>d</sup>	-	none	6	7 x 10 <sup>2</sup>
	+	none	5 <sup>e</sup>	2 x 10 <sup>2</sup>
	+	anti-YopM	6	5 x 10 <sup>2</sup>
	+	irrelevant	-	3 x 10 <sup>3</sup>
<i>L. monocytogenes</i> EGD	-	none	3	3 x 10 <sup>2</sup>
	+	none	3	5 x 10 <sup>2</sup>

<sup>a</sup> 100 µg of YopM were administered (IV or IP) in 100 µl of PF-PBS on days 0 through 5.

<sup>b</sup> 500 µg of protein A-purified rabbit antibody was administered IP in 500 µl of PF-PBS on days 0 and 2. Irrelevant antibody was purified from pre-immune serum samples.

<sup>c</sup> MTD - mean time to death (days) for mice given the dose that was at least 10-fold above the calculated LD<sub>50</sub> (10<sup>4</sup> bacteria).

<sup>d</sup> *Y. pestis* KIM5-3233 is a YopM<sup>-</sup> mutant.

<sup>e</sup> Not different at 95% confidence from MTD for *Y. pestis* KIM5-3233 without exogenous YopM

Discussion of passive and active immunization findings. In these experiments we sought to determine if immunization against YopM is protective against challenge with *Y. pestis*. Antibodies to YopM were hypothesized to be protective, because YopM has an in-vitro activity, thrombin-binding, that is compatible with an extracellular location for the protein during an infection and because YopM is necessary for full virulence in mice (2). Hence, antibody might have access to YopM in vivo and be able to neutralize its activity. To test our hypothesis, we used an intravenous challenge mouse model of systemic *Y. pestis* infection.

We found that YopM is highly immunogenic in mice and rabbits, and the tests with human convalescent sera indicate that humans also will respond to YopM produced during an infection with an antibody response.

However, neither passive immunization of mice with antibody from YopM-immunized mice or rabbits nor active immunization with YopM protected against *Y. pestis* challenge. In contrast, we found that both passive and active immunization against LcrV (HTV) was protective in our mouse plague model. As seen previously, active immunization against LcrV appeared to convey

strong protection (12,13), while passive immunization conveyed only partial protection (8), indicating that specific host factors, in addition to antibody, are required for full protection against *Y. pestis* challenge in mice.

It is possible that YopM is not neutralized by antibody because its main target in the host is intracellular. In vivo, YopM might enter host cells by the well-established vectorial translocation mechanism that functions for YopE, YopH, and YpkA and thereby be sequestered from access to antibody. Indeed, recent tests with chimeric proteins consisting of N-terminal portions of YopM fused to the adenylate cyclase domain of the *Bordetella pertussis* hemolysin-adenylate cyclase indicate that some of the chimeric YopM is translocated into cells. However, the fraction that was vectorially targeted in that study was relatively low (14). This leaves the significance of intracellularly targeted YopM unresolved and does not rule out an extracellular site of YopM function.

It is possible that YopM's virulence mechanism is not neutralizable by antibody, even though YopM might have an extracellular function. For example, if YopM has an environmentally modulated conformation, its putative active site might not be accessible to antibody because it is sequestered within the protein. We were intrigued by the finding that YopM could partially restore virulence to the YopM<sup>-</sup> *Y. pestis* strain without exacerbating a *Listeria* infection. This suggests that YopM in fact can have a virulence-related effect from an extracellular location but that the effect is *Yersinia*-specific - perhaps because YopM acts in concert with another *Yersinia* component. If added YopM needs to interact with yersiniae to exert its effect, the failure of exogenous YopM to restore complete virulence to the YopM<sup>-</sup> *Y. pestis* could have been due to the inability of the added YopM to reach foci of infection in sufficient concentration. Moreover, lethality is a relatively stringent readout of pathogenicity; we believe that the enhanced virulence of the YopM<sup>-</sup> *Y. pestis* in mice treated with YopM is significant and merits further study.

Our previous (3) and recent (detailed below) studies of thrombin-binding by YopM are consistent with a major virulence role of YopM other than thrombin-sequestration, as YopM's interaction with thrombin appears to be relatively weak. This does not rule out a possible locally high and effective YopM concentration at a focus of infection, but does raise the issue of possible other targets for YopM, including intracellular ones. In the experiments of Aim 3, we are examining the fates of exogenously added YopM and of YopM secreted by yersiniae to clarify the functions of this important *Yersinia* virulence protein.

Aim 2. Determine if thrombin-binding is necessary for YopM function in vivo.

TO 1. Identify thrombin-binding sites on YopM. and TO 2. Eliminate thrombin-binding to YopM by site-directed mutagenesis of yopM.

Introduction. A model has been proposed in which YopM significantly contributes to the virulence of *Y. pestis* by acting as an anti-inflammatory agent (15). YopM might function by sequestering thrombin. The resulting decreased clotting and inflammation would promote bacterial spreading and cripple the body's ability to deliver bactericidal cells to the focus of infection. To assess the significance of YopM's thrombin-binding in virulence, we plan to create and test the virulence of *Y. pestis* strains expressing mutant YopM molecules defective in thrombin-binding.

YopM belongs to a family of proteins that contain a leucine-rich repeat (LRR) motif (16). In porcine ribonuclease inhibitor, the prototype LRR protein for which X ray data are available, these tandem repeats create an extended "horse shoe"-like structure of parallel  $\beta$  strands connected by  $\alpha$ -helices (17, 18). YopM was predicted by Kobe and Deisenhoffer (16) to have 12 tandem repeats, however a potential 13th repeat at the beginning of the stretch of repeats can be arranged (Fig. 1). This one, however, has the conserved N shifted by one residue. YopM also has significant homology in its predicted sequence with the  $\alpha$ -chain of GPIb human platelet surface receptor. GPIb $\alpha$  also is an LRR family member, and its LRR structure was the basis of the homology to YopM. However, it is the only other LRR family member known to bind thrombin. The only commonality that emerges among the more than 40 LRR proteins is that they interact with other proteins, many functioning at the cell surface.

$\alpha$ -Thrombin is a serine protease with many structural similarities to trypsin but with a much greater specificity (19-21). Its active site is in a deep pocket partially occluded by two protruding loops that limit access of potential substrates. Moreover, thrombin's activity on protein substrates (as opposed to small-molecule synthetic substrates) requires binding of the substrate at one or more regions distinct from the catalytic site named "exosites". The catalytic pocket is flanked on the "west" and "east" by an apolar binding site and the anion-binding exosite I (abeI, also called the fibrinogen recognition exosite), respectively, and on the "north" by anion binding exosite II (abeII, the heparin binding site on thrombin). Abe I and II represent positively charged patches on the surface of thrombin (21, 22). Substrates (fibrinogen, thrombin receptor, coagulation factor VIII), most exogenous inhibitors (the leech anticoagulant hirudin, the sulfated dodecapeptide representing hirudin's C-terminal tail called hirugen, an oligonucleotide aptamer), and activity modulators (thrombomodulin) bind to abeI by means of linear peptide sequences having clusters of negatively charged

residues interspersed with hydrophobic residues that insert into the hydrophobic crevices of abeI. The endogenous thrombin inhibitor heparin cofactor II, following its interaction with heparin, also interacts with abeI. There is no strict consensus sequence for the abeI-binding regions of these molecules (20). Models for how they bind to abeI are based on information from the crystal structures of  $\alpha$ -thrombin bound to hirudin, to hirugen, and to peptides derived from the tethered-ligand thrombin receptor (23-26). Hirudin, a 65-residue peptide, has an N-terminal disulfide-knotted head that associates with the apolar site on thrombin and a C-terminal anionic tail. The tail binds in an extended conformation to abeI (24, 27).

MFINPRNVSNTFLQEPLRHSSN																	LTEMPV						
E	AENVKSKTEYYNAWSEWERNAPPGNGEQREMAVSRRLRDCCLR																						
72	Q	A	H	E	L	E	L	N	N	L	G	L	S	S	L	P	E	L	P	P	91		
92	H	L	E	S	L	V	A	S	C	N	S	L	T	E	L	P	E	L	P	Q	111		
112	S	L	K	S	L	V	D	N	N	N	N	L	K	A	L	S	D	L	P	P	131		
132	L	L	E	Y	L	G	V	S	N	N	Q	L	E	E	L	P	E	L	Q	N	L	P	153
154	F	L	T	A	I	Y	A	D	N	N	S	L	K	K	L	P	D	L	P	L			173
174	S	L	E	S	I	V	A	G	N	N	I	L	E	E	L	P	E	L	Q	N	L	P	195
196	F	L	T	T	I	Y	A	D	N	N	L	L	K	T	L	P	D	L	P	P			215
216	S	L	E	A	L	N	V	R	D	N	Y	L	T	D	L	P	E	L	P	Q			235
236	S	L	T	F	L	D	V	S	E	N	I	F	S	G	L	S	E	L	P	P			255
256	N	L	Y	Y	L	N	A	S	S	N	E	I	R	S	L	C	D	L	P	P			275
276	S	L	E	E	L	N	V	S	N	N	K	L	I	E	L	P	A	L	P	P			295
296	R	L	E	R	L	I	A	S	F	N	H	L	A	E	V	P	E	L	P	Q			316
316	N	L	K	Q	L	H	V	E	Y	N	P	L	R	E	F	P	D	I	P	E			335
S	EDLRMNSERVVDPYEF AHETTDKLEDDVFE																						

Figure 1. Amino-acid sequence of YopM. Residues 72-335 are arranged in 13 rows, where each row is a leucine rich repeat (LRR). LRR conserved residues are marked by vertical boxes. Regions within horizontal boxes resemble the abeI-binding C-terminus of hirudin. Numbers on both sides show the residues of LRR repeats.

In Year 1 of this project, we showed that purified YopM binds to  $\alpha$ -thrombin at abeI or a region very near it and also interacts with thrombin's catalytic site. It is not hard to find sequences resembling hirudin's abeI-binding region in the acidic YopM: seven of these are indicated in Fig. 1. One, and perhaps two of these were tentatively eliminated by our work in Year 1. Antibodies raised against a peptide (LEELPELQNLEF) corresponding to 2 exactly repeated hirudin-like sequences (LEELPEL) (Fig. 1) recognized the crosslinked complex. In addition, the synthetic peptide used to raise the antibodies did not inhibit complex formation. The other hirudin-like domains are candidates for the

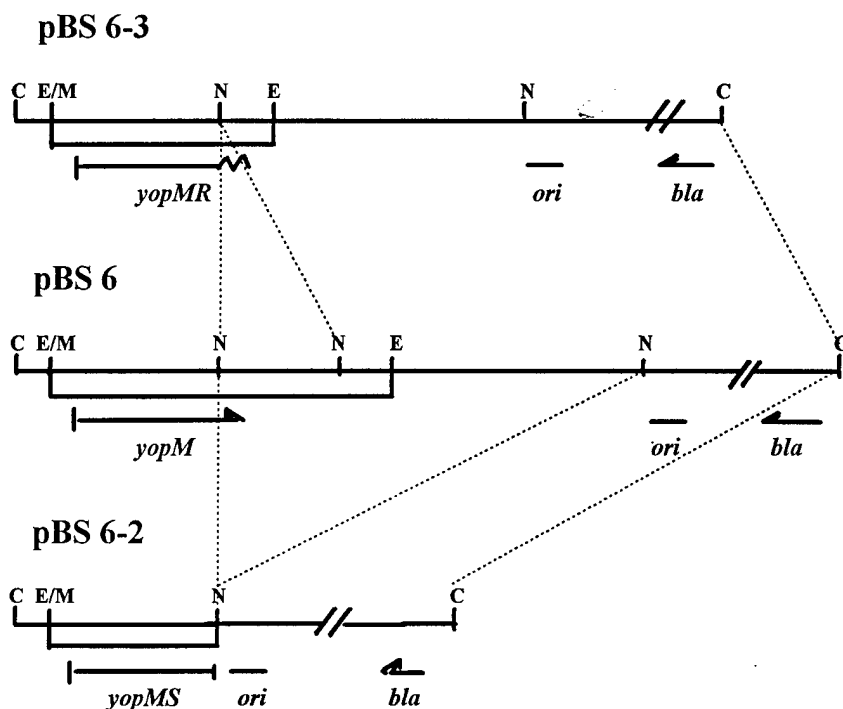
site where the interaction with *abeI* occurs. In addition, YopM residues 206-227 potentially can form a leucine zipper that could mediate protein-protein interactions (32). We are testing these possibilities by creating recombinant YopM molecules lacking these regions but not disrupted in their potential  $\beta$ -sheet structure.

Outcome of tests in progress at the end of year 1. We had made two constructs that encoded histidine-tagged halves of YopM containing residues 1-142 and 143-368, respectively, of YopM (HIS-YopM 1-142 and HIS-YopM 143-368). The goal was to test these for their ability to bind thrombin and thereby determine if YopM's thrombin-binding site lay in the N- or C-terminal half of the protein. These proteins were poorly expressed, and we were not sure that we would be able to get enough for the tests. We did try to produce enough of these proteins by growing large cultures of *E. coli* containing the constructs, but found it difficult to obtain enough of the cleaved fusion protein to make a convincing test. The problem seemed to be that the fusion protein adhered nonspecifically to the affinity resin and was not recovered. It became clear that it was not practical to take this route, the more so because their marginal solubility was a red flag: both proteins might be abnormally folded.

We also had two fusion constructs that contained YopM's C-terminus (GST-YopM 260-368 and HIS-YopM 288-368), and these were soluble and cleavable from the fusion partner by Factor Xa and TEV protease, respectively. In one competitive crosslinking test, these did not inhibit YopM's binding to thrombin. We discontinued working with fusion constructs and took a new approach, the construction of *yopM* genes having specific deletions.

Construction of *yopMS* and *yopMR*. To test the importance for thrombin-binding of the C-terminal 16 YopM residues, containing 7 acidic (D,E) and one basic (K; or 2 basic, if H is counted) amino acids, we made two constructs: YopMS was missing these and had two hydrophobic residues (A,V) added at the C-terminus, and YopMR replaced the C-terminal 15 residues with 14 irrelevant residues encoded by the pBR322 vector. The construction is illustrated in Figure 2, with the the C-terminal sequences detailed in the bottom panel. The *yopM*-containing plasmid pBS-6 (28) was used for the constructions because it overexpresses YopM, probably because it lacks an upstream attenuating sequence (3). This plasmid has the same cloned *Yersinia* DNA as does pBS7 (3) but oriented oppositely. It contained 3 *NdeI* sites, one near the end of *yopM* and two within the vector, the third being near the vector's origin. YopMS resulted from cleavage at the first and third *NdeI* sites and religation; YopMR resulted from cleavage at the first and second sites.

A)



B)

#### YOPM (pBS6)

Nde I  
 CA TAT GAA TTT GCT CAT GAG ACT ACA GAC AAA CTT GAA GAT GAT GTA TTT GAG TAG  
 Y E F A H E T T D K L E D D V F E STOP

#### YOPMR (pBS6-3)

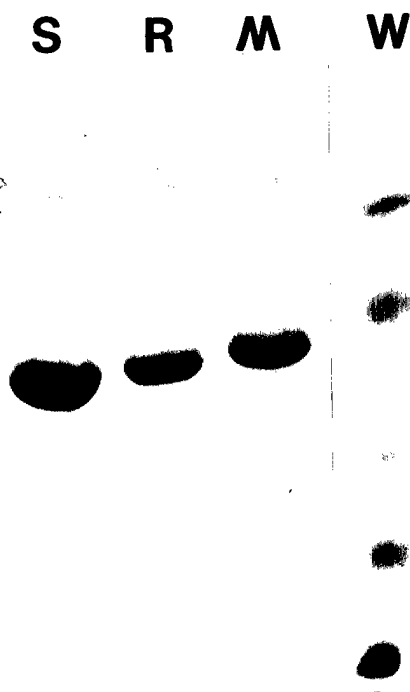
Nde I  
 CA TAT GAG TAT TCT CCG ATT ACA TAT ATG TTA GTT GCC ATG GAT AAT CCG TAA  
 Y E Y S P I T Y M L V A M D N P STOP

#### YOPMS (pBS6-2)

Nde I  
 CA TAT GCG GTG TGA  
 Y A V STOP

Figure 2. Construction of YopMS and YopMR. The genetic manipulations are represented in panel A: the plasmid pBS-6 was cleaved with NdeI and religated. YopMR and S resulted from cleavages at the site within *yopM* and at the proximal and distal other two sites, respectively. Restriction enzyme sites are: E, EcoRV; M, MscI; N, NdeI; C, ClaI. Panel B shows the C-terminal sequences of the resulting genes and predicted proteins.

Characterization of YopMS and YopMR. Plasmids pBS6-2 and pBS6-3 carrying *yopMS* and *yopMR* were transformed into a YopM<sup>-</sup> strain of *Y. pestis* KIM that was lacking the pPCP1 plasmid that encodes the Pla protease that degrades Yops. The resulting strains were grown as 3.2-1 cultures, induced for Yops expression and the YopM proteins isolated as previously described (3). YopMS and R were strongly expressed and secreted. Because YopM is acidic throughout its length, these truncated YopMs had isoelectric points similar to YopM (e.g., that predicted for YopMS was 4.12 as compared to 4.06 for YopM), and these proteins purified similarly to YopM upon anion exchange chromatography. Figure 3 shows that the resulting proteins were comparable in purity to our best YopM preparations.



**Figure 3. Purified YopMS and YopMR.** Samples of purified YopM, YopMS, and YopMR were analyzed by SDS-PAGE in PhastSystem (Pharmacia LKB, Uppsala Sweden) 12 % acrylamide gels. Bands were visualized by staining with Coomassie Brilliant Blue. Lanes: S, YopMS; R, YopMR; M, YopM; W, molecular weight standards.

Figure 4 shows the results of a chemical crosslinking analysis using disuccinimidyl suberate (DSS) as previously described (3,29). Both YopMS and R bound to thrombin and could be crosslinked to form the ca. 75 kDa complex (arrowhead in Fig. 4). YopMR may have interacted slightly less efficiently than did YopM, while YopMS consistently appeared to bind thrombin more tightly than does YopM, giving more crosslinked product. The



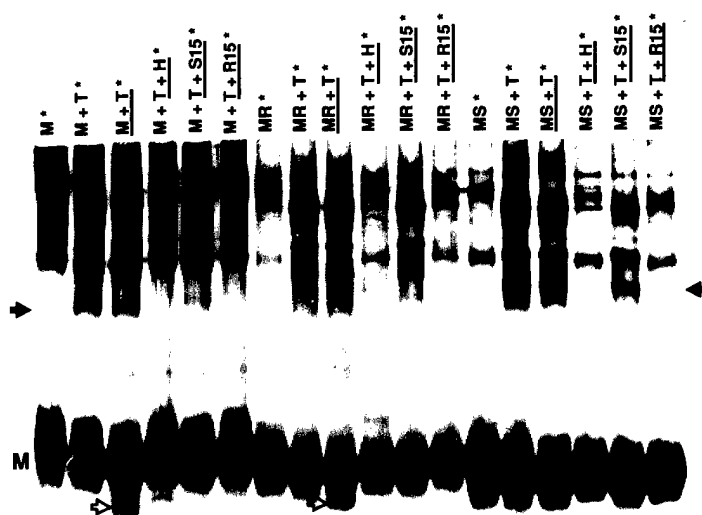


Figure 4. YopMS and YopMR both bind thrombin. Proteins were separated by SDS-12% (wt/vol) acrylamide PAGE, and immunoblots were probed with polyclonal rabbit antibodies raised against the whole YopM molecule. YopM (M), YopMS (MS), or YopMR (MR) was preincubated (underline) or not with  $\alpha$ -thrombin (T) before crosslinker DSS (\*) was added. Competitors: hirudin (H) or aptamers (R15, S15). The positions of noncrosslinked YopM and truncated YopMs is marked at the left by M, the crosslinked product containing one YopM and one thrombin is indicated by a closed arrow, and a truncated YopM due to cleavage by thrombin is indicated with an open arrowhead. Higher-molecular weight products represent YopM dimer and perhaps also trimer and such forms with thrombin bound (previous annual report and ref. 28).

binding of both YopMS and YopMR to thrombin was at thrombin's abel, because it was outcompeted by hirudin and the aptamer R15. The interaction was specific, because it was not outcompeted by the scrambled aptamer S15. These data show that the C-terminal-most amino acids of YopM are not necessary for thrombin-binding; if anything, they make the interaction with thrombin less efficient. The data also show that, as with YopM, when YopMR was preincubated with thrombin for 20 min prior to the crosslinking reaction, thrombin was able to cleave off a small C-terminal fragment, leaving a truncated cleavage product (open arrowhead). Because YopMS was already C-terminally truncated, it was not possible to tell in whether it was slightly further

truncated by thrombin. Together, these data indicated that YopMR and YopMS interacted essentially normally with thrombin, and we did not study them further. It should be noted, however, that YopMS can be useful in studies where it is important to distinguish in immunoblots the YopM products of two *yopM* genes in the same strain, e.g., *yopM* in the LCR plasmid under LCR control and *yopMS* in trans on a plasmid and expressed from a heterologous regulatable promoter such as the IPTG-inducible *P<sub>trc</sub>*.

#### Construction of *yopM* genes expressing internally deleted YopMs.

General strategy. We wanted to test whether various LRRs of YopM are responsible for thrombin-binding, but wanted to avoid grossly deranging the protein's structure. Although we do not know YopM's 3° structure, we used its LRR architecture as a basis for a rough model for how YopM might appear if it followed the pattern of porcine RNase inhibitor (Figure 5). The resulting picture revealed the possibility of remarkable distribution of charge within YopM: residue 17 of each 20- or 22-residue LRR is negatively charged and predicted to be exposed on the outside of the protein. These residues would make the "bottom" of the molecule very negatively charged. The top outside also would be charged around most of the protein, due to residues # 11, 13, and 14 of each LRR; however, in various LRRs these may differ. The least charge at the top of the molecule as drawn in Fig. 5 is in the vicinity of the putative leucine zipper (LRRs 7 and 8). Because the protein probably is highly structured, we felt it important to preserve its LRR arrangement and not delete part of an LRR, which might make an unaccommodated kink in the molecule. Moreover, because some LRRs alternated in the presence of + and - charge in the connecting strands (LRRs 3 through 7), we were reluctant to disrupt that pattern. Accordingly, we chose to delete pairs of LRRs.

To make the deletions, we followed the strategy of Wren et al. (30) and designed primers in opposite orientations at a gap that defined the deletion to be made in *yopM* (Figure 6). The 5' end of each primer contained the *yopM* sequence altered by 1 or 2 bases so as to create a restriction site for a blunt-end-creating restriction enzyme without changing the amino acids normally encoded by that part of *yopM*. A PCR reaction would then initiate at both primers, extending in opposite directions and running

Top

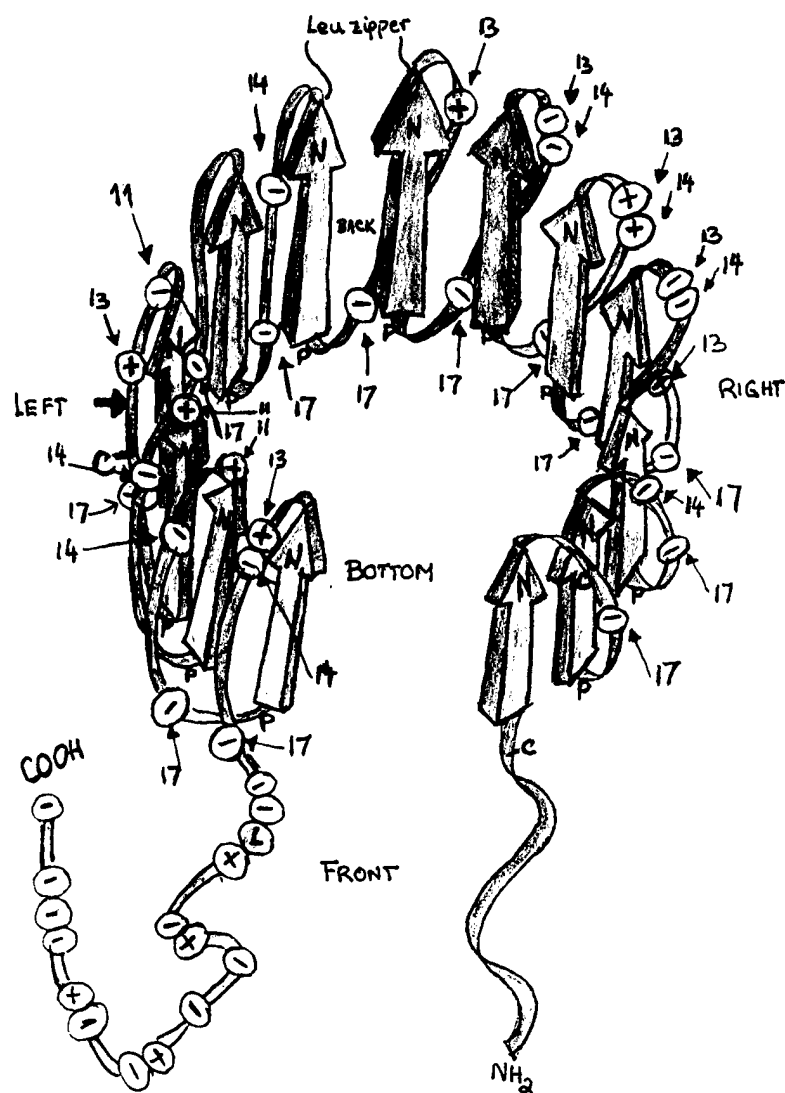
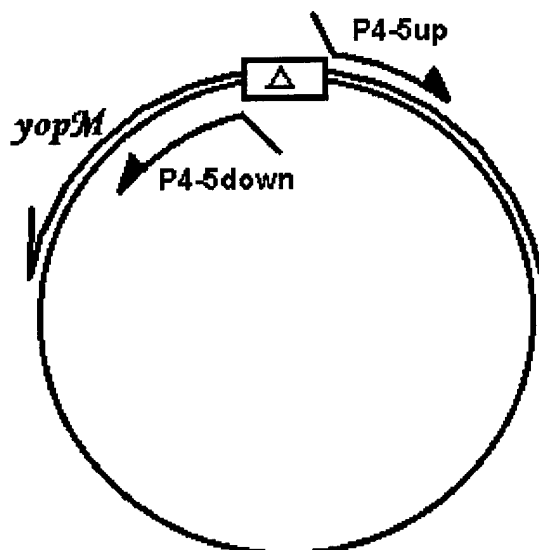


Figure 5. Cartoon of YopM based on the assumption that YopM's arrangement resembles that of porcine RNase inhibitor. Each of the 13 LRRs is depicted by an arrow representing a  $\beta$ -sheet element and a ribbon connecting the C-terminus of the  $\beta$ -sheet to the N-terminus of the adjacent parallel  $\beta$ -sheet element of the next LRR. Negatively charged residues are indicated by -, positively charged residues by +, some prolines by P, the conserved asparagines by N (located at the C-termini of the  $\beta$ -sheet elements), and cysteines by C. Within each 20 or 22-residue LRR, some residues are indicated by number, where the numbering begins with 1 as the N-terminal-most residue in the LRR.

around the entire plasmid template. The PCR products were digested with the restriction enzyme for which sites were present in the primers and religated. This created plasmids containing the desired deletion within *yopM*. The plasmids were transformed into *E. coli* XL1 Blue with selection for the ampicillin-resistance on the pBR322-derived vector (100 µg/ml ampicillin) and were reisolated from selected colonies for confirmation of their restriction maps.



**Figure 6. General strategy for creating deletions within cloned *yopM*.**

Details of the constructions.

Construction of pBS15/15. We modified pBS15 (3), which includes ca. 780 bp of DNA upstream of *yopM*, for our deletion work. Because the PCR reactions had to extend through the entire plasmid, we first made the plasmid smaller. We deleted most of this upstream sequence by cleaving with *MscI* and *Bst* 1107I and religating. This created pBS15/15, 3699 bp in size. It also converted the plasmid from one that does not overexpress YopM to one that does by removing the upstream sequence that down-modulates *yopM* expression (3).

Construction of specific deletions. The primer pairs used for the deletions are given in Table 4. Figure 7 illustrates a specific example of one construction using the primer pair designed to delete LRRs 4 and 5. The PCR reactions employed a Perkin-Elmer Cetus GeneAmp PCR System 2400 thermocycler and the following conditions: for LRRs 1-2, 4-5, 6-7, and 7-8, 94°C 5 min (hot start); then 40 cycles of: 94°C 1 min, 45°C 1 min, 72°C 4 min; then 72°C 15 min; then storage at 4°C. For LRRs 4-7 and 7-13, the conditions were 94°C 7 min (hot start); then ca. 40

Table 4. Primers for the deletion of LRRs in YopM.

Primer	Sequence and Restriction Site
<u>LRRs 1-2; Obtained ALRRs 1-7</u>	
	<i>AflIII</i>
P1-2 down	5'AGG TAC <u>CTT AAG</u> GCT TCG GTC CAG GCA ATC TCG TAA CCT TGA 3'
	<i>AflIII</i>
P1-2 up	5'GTAC AGC <u>CTT AAG</u> TCA CTT CTA GTT GAT AAT AAC AAT CTG AAG GCA 3'
<u>LRRs 4-5; Obtained: ALRR4-5</u>	
	<i>Sma I</i>
P4-5 down	5'TACG <u>CCC GGG</u> TAA ATC GGA TAA TGC CTT CAG ATT 3'
	<i>Stu I</i>
P4-5 up	5'TACG <u>AGG CCT</u> TCA CTG GAA TCT ATT GTT GCT GGT 3'
<u>LRRs 6-7; Obtained: ALRR6-7, ALRR4-7, ALRR2-7, and ALRR7</u>	
	<i>Sma I</i>
P6-7 down	5'TACG <u>CCC GGG</u> TAA ATC AGG TAG TTT TTT CAG TGA ATT 3'
	<i>Ecl136II</i>
P6-7 up	5'TACG <u>GAG CTC</u> TCC CTG GAA GCA CTT AAT GTC AGA 3'
<u>LRRs 7-8; Obtained: ALRR7-8 and ALRR 5-8</u>	
	<i>Sma I</i>
P7-8 down	5'GATC <u>CCC GGG</u> AG GTT TTG CAA CTC TGG CAA 3'
	<i>EcoRV</i>
P7-8 up	5'GATC <u>GAT ATCC</u> TTA ACC TTC TTA GAT GTT TCT GAA AAT ATT 3'
<u>LRRs 4-7; Obtained: ALRR 4-7</u>	
P4-5 down	
P6-7 up	
<u>LRRs 7-13; Obtained: ALRR 7-13</u>	
P7-8 down	
	<i>EcoRV</i>
P12-13 up	5'GATC <u>GAT ATC</u> GG TGG AA GAT CTT CGG ATG AAC TCT 3'
<u>NH<sub>2</sub>(aa19) - LRR4; Obtained NH<sub>2</sub>-5</u>	
	<i>SmaI</i>
PNH <sub>2</sub> down	5'TGC TAG <u>CCC GGG</u> TTC TTG CAA AAA AGT ATT AGA TAC ATT TCT 3'
P4-5 up	
<u>NH<sub>2</sub>(aa19) - LRR6; Obtained NH<sub>2</sub>-ca.6, NH<sub>2</sub>-8, and NH<sub>2</sub>-ca.9</u>	
PNH <sub>2</sub> down	
P6-7 up	

```

    92  H L E S L V A S C N S L T E L P E L P Q      111
    112 S L K S L L V D N N N L K A L S D L P P      131
    132 L L E Y L G V S N N O L E E L P E L Q N L P 153
    154 F L T A I Y A D N N S L E K L P D L P L      173
    174 S L E S I V A G N N I L E E L P E L Q N L P 195
    196 F L T T I Y A D N N L L K T L P D L P P      215

```

Actual yopM sequence:

```

GAT  TTA  CCA  CCT /Δ4-5LRR/  TCA  CTG  GAA  TCT
D    L    P    P131           S174 L    E    S

```

Primer sequences (SmaI and StuI sites are created):

```

GAT  TTA  CCC  GGG
CTA  AAT  GGG  CCC-5' P4-5 down
D    L    P    P    ←

```

```

→
P4-5 up  5'AGG  CCT          TCA  CTG  GAA  TCT
          P

```

After PCR, cleavage with SmaI and StuI, and blunt-end ligation (| = point of ligation):

```

GAT  TTA  CCC|CCT /Δ4-5LRR/  TCA  CTG  GAA  TCT
D    L    P    P           S    L    E    S

```

Figure 7. Creation of ΔLRR4-5. The relevant portion of the YopM amino acid sequence is shown at top. The example illustrates how the mutagenic primers create restriction sites while retaining the YopM amino acid sequence and prime to make a product, which when digested at the new sites and religated, makes the desired in-frame deletion.

cycles of: 94°C 1 min, 55°C 1 min, 72°C 1 min; then 72°C 20 min; then storage at 4°C. Because of the highly repetitive nature of *yopM* (15), it was difficult to identify primers that would not prime at multiple sites. Some of our primers produced a family of deletions, and some produced unexpected deletions (Table 4). Both of these events expanded the array of products for analysis. Figure 8 diagrams the constructs obtained from the deletion mutagenesis. In several cases, the endpoints of the deletions are not known yet. Ones that prove to be informative will be sequenced across the fusion junction, but this has not been done yet.

The constructs were transformed into the YopM<sup>-</sup> Pla<sup>-</sup> Y. *pestis* KIM and grown as we did for the YopMS and YopMR constructs, to express the mutant YopM proteins. All constructs except the series deleting after residue 18 (denoted NH<sub>2</sub>-x) strongly produced their internally deleted proteins. The NH<sub>2</sub>-9 construct did not produce a recognizable YopM protein. This will not be studied further. The NH<sub>2</sub>-ca.6, NH<sub>2</sub>-ca.8, and NH<sub>2</sub>-ca.4 constructs do make their protein but not strongly. They will be

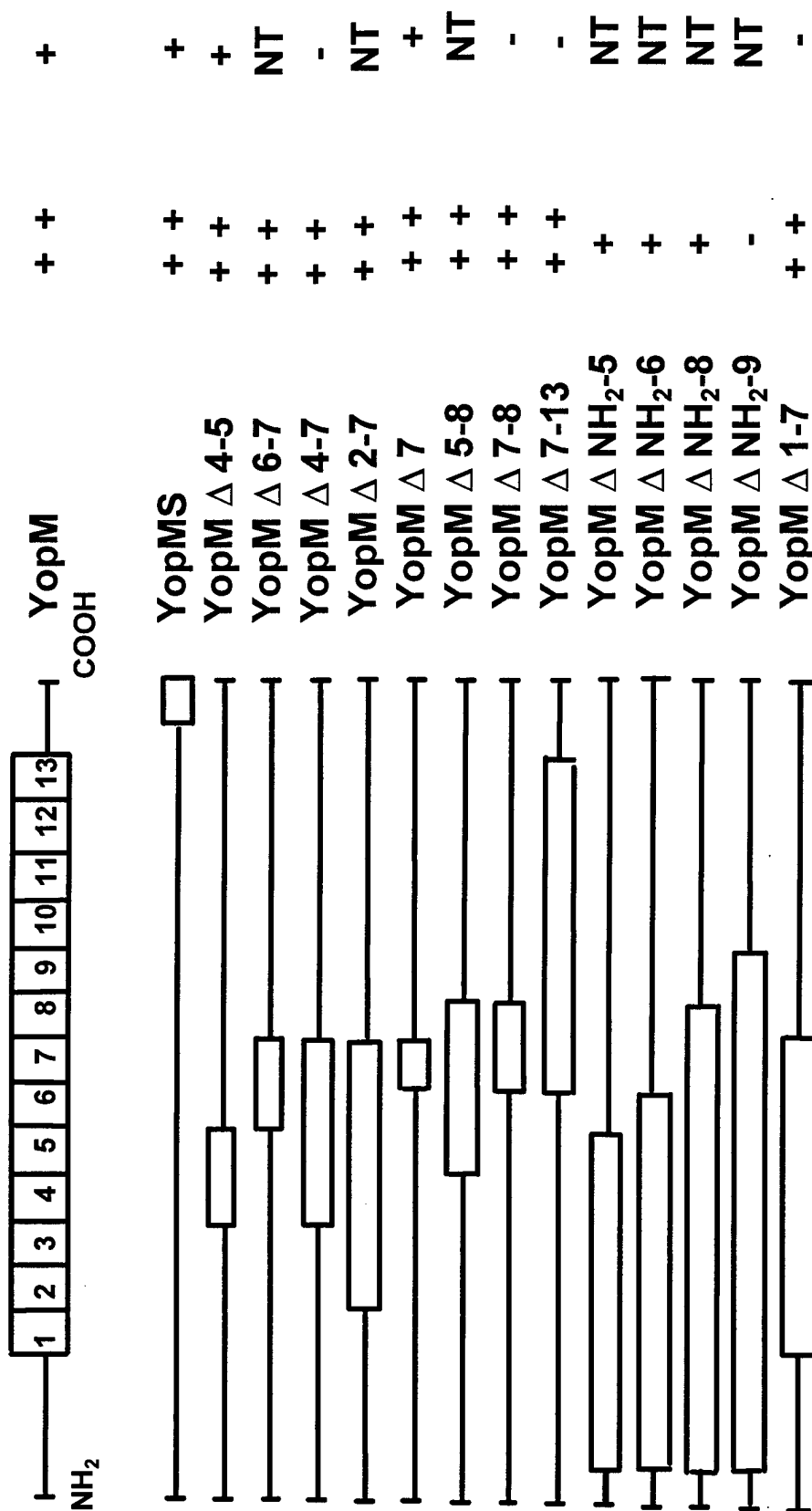


Figure 8. YopM deletion constructs. The top diagram represents YopM, with the 13 LRRs boxed. The lines indicate the cloned yopM DNA, and the extents of each deletion are indicated below by the boxes. Their strength of internally deleted YopM expression is indicated by - to 2+, and the abilities of the purified protein to bind  $\alpha$ -thrombin and form a crosslinked product (C-X) are indicated by + or -. NT = not tested. YopM7 is suspected by its size to have an unexpected structure.

set aside and studied further only if they are needed. The others are being grown in 1.6-l cultures to produce and purify their YopM in sufficient amounts for analysis of thrombin-binding and for experiments of Aim 3. So far, this has been done for YopMA1-7, YopMA4-5, YopMA4-7, YopMA7, YopMA7-8, and YopMA7-13. Figure 9 shows their protein profiles after purification on DEAE Sephacel ion exchange chromatography. Note that the profiles of these purified YopMALRRs resemble that for similarly loaded YopM, except for their size. This, plus their strong expression and normal secretion by *Y. pestis*, indicates that these YopMALRRs are not grossly deranged or targets for proteolysis. Not prepared yet are: YopMA2-7, YopMA6-7, and YopMA5-8. We plan to purify YopMA6-7, as it will provide useful information (see below), but will purify the other two only if they are needed, as we already have several constructs that appear to adequately cover this amino-terminal half of YopM.



**Figure 9. Profiles of purified YopMALRR proteins.** Purified proteins were analyzed by SDS-PAGE in PhastSystem 12% acrylamide gels. Bands were visualized by staining with Coomassie Brilliant Blue. Left panel, lanes: B = BSA, 7-8 = YopMALRR7-8, 1-7 = YopMALRR1-7, 4-7 = YopMALRR4-7. Middle panel, lanes: 7-13 = YopMALRR7-13, B = BSA, W = molecular weight standards. Right panel, lanes: 4-5 = YopMALRR4-5, 7-8 = YopMALRR7-8, M = full-length YopM, B = BSA, W = molecular weight standards. (NOTE: YopMALRR7 is not shown in this figure, but it was purified and is shown in Fig. 10.)

We tested the six purified YopMALRR proteins for their ability to bind  $\alpha$ -thrombin, using crosslinking by DSS as the assay. The findings are summarized in Figure 8. Figure 10 gives examples of the data and shows that two YopMALRR proteins retain



the ability to bind thrombin. These two, YopM $\Delta$ 4-5 and YopM $\Delta$ 7, appeared to interact normally with thrombin, forming the 1:1 crosslinked product with thrombin (as well as the characteristic spectrum of higher molecular weight forms normally seen in these assays) and being cleaved near the C-terminus by thrombin. None of the other four YopM $\Delta$ LRRs bound thrombin; an example of a negative reaction is given in Figure 10 for YopM $\Delta$ 4-7. Interestingly, this YopM $\Delta$ LRR nonetheless was cleaved by thrombin. This indicates that thrombin can act as a protease and cleave YopM independently of the specific interaction at *abeI*. Evidently this proteolysis interaction is not long-lived enough to allow crosslinking of YopM to thrombin; alternatively, the interaction juxtaposes the two proteins without bringing crosslinkable free amines near enough to be linked by DSS. In either model, the interaction leading to proteolysis appears to be distinct from the binding of YopM at *abeI*.

The data show that LRRs 4 and 5 of YopM are not necessary for the specific binding of thrombin at the fibrinogen-binding site, *abeI*. We do not know the exact location of the deletion in YopM $\Delta$ 7. That construct was made with primers designed to delete both LRRs 6 and 7, but by its size, it contains a smaller deletion. LRRs 7 and 8 contain a putative leucine zipper structure in YopM (see Fig. 5), at residues 206-227. All of our YopM $\Delta$ LRR proteins that failed to bind thrombin (YopM $\Delta$ 1-7, YopM $\Delta$ 4-7, YopM $\Delta$ 7-8, and YopM $\Delta$ 7-13) had in common the deletion of LRR 7. The most conservative interpretation of the data is that YopM's thrombin-binding region begins at LRR ca. 7 and extends toward the C-terminus. At the minimum, LRRs 7 and 8 are required. We are preparing to test YopM $\Delta$ 6-7, which will rule more closely on the importance of LRR7, and we are sequencing constructs YopM $\Delta$ 4-5, YopM $\Delta$ 7, YopM $\Delta$ 6-7, and YopM $\Delta$ 7-8 to learn their precise deletion endpoints. These 4 constructs are particularly interesting, as they sequentially probe the region of LRRs 4 through 8 with relatively small deletions, and two of the protein products bind thrombin, while two do not.

Our goal is to identify a small deletion that abolishes thrombin-binding without grossly deranging the protein and then to move this mutation into *pCD1* in *Y. pestis* and test virulence. If virulence is not changed by the mutation, this would disprove the hypothesis that thrombin-binding is YopM's main function in virulence. If such a *Y. pestis* YopM $\Delta$ LRR mutant is as avirulent as is YopM- *Y. pestis*, this would be consistent with the hypothesis or alternatively indicate that whatever other function YopM has in vivo, it requires regions of YopM in common with that needed for thrombin-binding. This will complete the work of Aim 2. Accordingly, in the coming year, we will move one or more of these four mutations into *pCD1* of *Y. pestis* and test the virulence of the resulting strain. The construct used to make the allelic exchange for introduction of the mutation into *pCD1* will be provided to Col. Friedlander's research group in fulfillment of the final deliverable of this Collaborative Agreement.

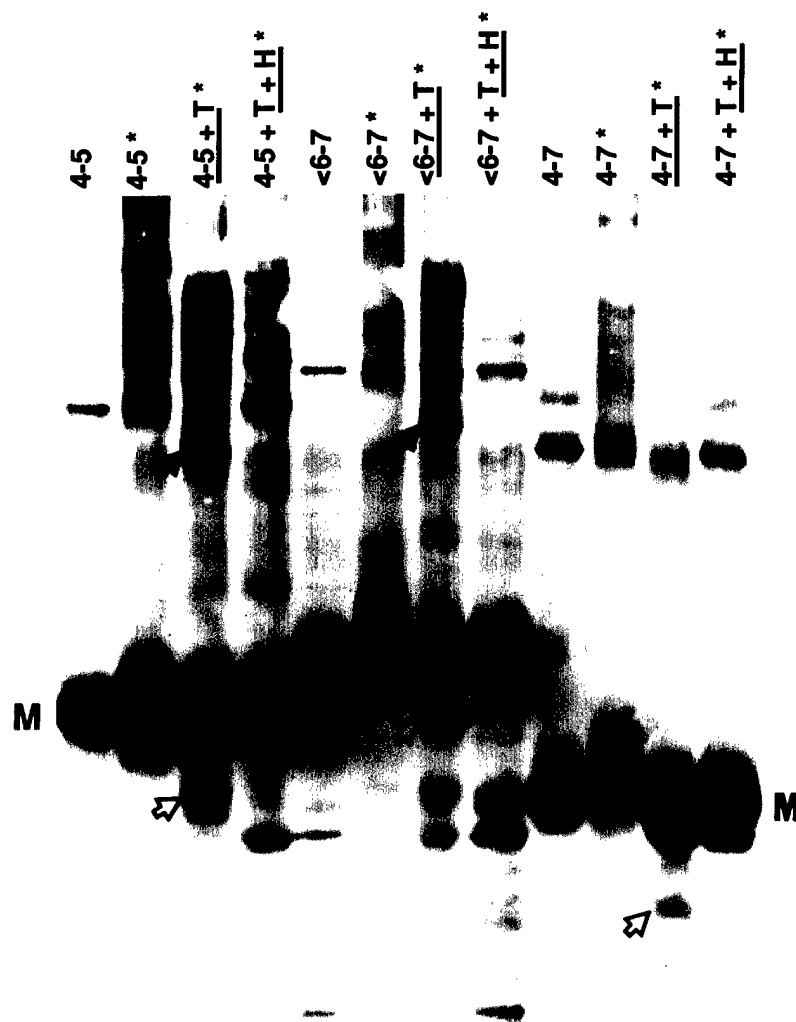


Figure 10. Thrombin-binding by three of the YopMALRR proteins. Proteins were separated by SDS-12% (wt/vol) acrylamide PAGE, and immunoblots were probed with polyclonal rabbit antibodies raised against the whole YopM molecule. YopM (M), YopMALRR4-5 (4-5), YopMALRR7(<6-7), or YopMALRR4-7 was preincubated (underline) or not with  $\alpha$ -thrombin (T) before crosslinker DSS (\*) was added. In one assay for each YopMALRR, the competitor hirudin (H) was preincubated with thrombin before the YopMALRR protein and then crosslinker were added. For each data set, the positions of noncrosslinked YopM and YopMALRRs is marked by M, the crosslinked product containing one YopM and one thrombin is indicated by a closed arrow, and a truncated YopM due to cleavage by thrombin is indicated with an open arrow. Higher-molecular weight products represent YopM dimer and perhaps also trimer and such forms with thrombin bound (Year 1 annual report and ref. 28).

Aim 3. Determine the fate of YopM when *Y. pestis* interacts with phagocytic cells.

To 1 and 2: Fate of YopM when *Y. pestis* attaches to the surface of a phagocyte and when *Y. pestis* is engulfed by a phagocyte.

To obtain information on YopM's virulence mechanism we need to identify where YopM localizes when *Y. pestis* infects a macrophage or other eucaryotic cell. Because the findings from our work on Aim 1 indicate that YopM may have a virulence role from an extracellular location as well as possibly when *Y. pestis* binds to eucaryotic cells, we are determining the fate of exogenously added YopM as well as YopM delivered by yersiniae through their Yop secretion mechanism.

Setting up the system: the yersiniae. To see if there is a difference in YopM targeting by yersiniae bound at the eucaryotic cell surface vs. by yersiniae that have been engulfed by macrophages (TO 1 vs. 2), we need to be able to set up assays in which the bacteria differ significantly in their surface vs. intracellular locations. We have created a series of *Y. pestis* KIM strains that vary in their adhesiveness (by the presence or absence of pAMS2 [33], which encodes the *Y. pseudotuberculosis* *yadA*) as well as in their strength of YopM expression (by having an insertion mutation in *yopM* and thereby being YopM<sup>-</sup> or by having the multicopy YopM-overexpressing plasmid pBS10 [3]). We use both *Y. pestis* KIM5 and Pla<sup>-</sup> *Y. pestis* KIM (officially termed *Y. pestis* KIM8). *Y. pestis* KIM8 is pigmentation-negative as is *Y. pestis* KIM5, and the absence of Pla minimizes degradation of any Yops released into the extracellular medium these in-vitro assays. These strains are listed below.

<u>Key properties</u>	<u>Strain</u>	<u>Comments</u>
YopM <sup>-</sup>	<i>Y. pestis</i> KIM5-3233	
YopM <sup>+</sup>	<i>Y. pestis</i> KIM5	
YopM <sup>+</sup> YadA <sup>+</sup>	<i>Y. pestis</i> KIM5 (pAMS2)	
YopM <sup>+++</sup> YadA <sup>+</sup>	<i>Y. pestis</i> KIM5 (pBS10 pAMS2)	
YopM <sup>-</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8-3233	(we have this strain with and without the pAMS2181 vector part of pAMS2)
YopM <sup>-</sup> YadA <sup>+</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8 (pAMS2)	
YopM <sup>+</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8	(we have this strain with and without pAMS2181)
YopM <sup>+</sup> YadA <sup>+</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8 (pAMS2)	
YopM <sup>++</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8-3233	(YopM <sup>-</sup> mutant with pBS10)
YopM <sup>++</sup> YadA <sup>+</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8-3233 (pAMS2)	(YopM <sup>-</sup> mutant with pBS10 and pAMS2)

In some tests, we also employ *Y. pseudotuberculosis*, as a reference, because Yops targeting previously has been studied in *Y. pseudotuberculosis* and *Y. enterocolitica* but not *Y. pestis*. We monitor the fate of YopM by immunofluorescence with conventional or confocal microscopy or by western analysis of fractionated infected eucaryotic cell cultures (see below). YopE is followed in the same way and serves as a reference protein known to be targeted into eucaryotic cells by yersiniae attached at the surface (33-35). The antibody we are using to detect YopE in these experiments was provided by Maj. Andrews of Col. Friedlander's group at USAMRIID. He also sent us some antibody to YopH, which similarly has been studied for its targeting into eucaryotic cells (36). We have made and hold in reserve a second set of positive and negative control strains if we need to vary the expression of YopE or YopH. This second set of strains varies in adhesiveness by having or not having pAMS2 (YadA+) and in YopE or YopH expression by having an insertion mutation in YopE or in YopH (to abolish expression of YopE or YopH) or by containing pYopE which encodes YopE and its specific Yop chaperone Syce (a gift of Greg Plano, University of Miami; this plasmid provides multicopy *yopE* and hence YopE overexpression). The *Y. pseudotuberculosis* strains and available additional *Y. pestis* strains are:

<u>Key properties</u>	<u>Strain</u>	<u>Comments</u>
YopM <sup>+</sup> YadA <sup>+</sup> Pla <sup>-</sup>	<i>Y. pseudotuberculosis</i> PB1/+	wildtype
YopM <sup>+</sup> YopE <sup>++</sup> YadA <sup>+</sup> Pla <sup>-</sup>	<i>Y. pseudotuberculosis</i> PB1/+ (pYopE)	
YopM <sup>+++</sup> YadA <sup>+</sup> Pla <sup>-</sup>	<i>Y. pseudotuberculosis</i> PB1/+ (pBS10)	
YopM <sup>+</sup> YopE <sup>-</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8-3122	
YopM <sup>+</sup> YopE <sup>-</sup> YadA <sup>+</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8-3122 (pAMS2)	
YopM <sup>+</sup> YopE <sup>+</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8-3122 (pYopE)	(YopE <sup>-</sup> mutant with multicopy <i>yopE</i> )
YopM <sup>+</sup> YopE <sup>+</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8-3122 (pYopE pAMS2)	(YopE <sup>-</sup> mutant with multicopy <i>yopE</i> )
YopM <sup>+++</sup> YopE <sup>-</sup> YadA <sup>+</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8-3122 (pBS10 pAMS2)	
YopM <sup>+</sup> YopE <sup>++</sup> YadA <sup>+</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8 (pYopE pAMS2)	
YopM <sup>-</sup> YopE <sup>++</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8-3233 (pYopE)	
YopM <sup>-</sup> YopE <sup>++</sup> YadA <sup>+</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8-3233 (pYopE pAMS2)	
YopM <sup>+</sup> YopH <sup>-</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8-3169	
YopM <sup>+</sup> YopH <sup>-</sup> YadA <sup>+</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8-3169 (pAMS2)	
YopM <sup>+++</sup> YopH <sup>-</sup> YadA <sup>+</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8-3169 (pBS10 pAMS2)	
YopM <sup>+</sup> YopE <sup>++</sup> YopH <sup>-</sup> Pla <sup>-</sup>	<i>Y. pestis</i> KIM8-3169 (pYopE)	

At this writing, we have not established one set protocol and still are optimizing the assays. However, we have identified some key parameters for the assays. We are using two eucaryotic cell lines, the J774 murine macrophage-like cell and the human epithelial line HeLa. The macrophage is a cell that normally is efficiently invaded by, or actively engulfs, *Y. pestis* (e.g., 37-

39), and Yops delivery has been shown to inhibit phagocytosis (e.g., 40,41). The HeLa cell line is a useful model for studying targeting of Yops, because it is a relatively flat cell and permits good visualization of distributions of proteins detected by fluorescent antibody techniques. It also has been used extensively in studying Yops targeting by *Y. pseudotuberculosis* (e.g., 33-36). We are using this cell line as a reference condition and because *Y. pestis* does not invade HeLa cells efficiently: HeLa cells provide a model in which almost all of the yersiniae in our experiments will be extracellularly located. We have tried two kinds of experiments. 1), pure YopM is added to J774 cells grown as adherent monolayers or as suspension cultures or to adherent HeLa cells, and after washing, the distribution of YopM is determined by fluorescent antibody visualized with UV epifluorescence or by laser scanning confocal microscopy using a Molecular Dynamics Multiprobe 2001 confocal microscope. 2) *Y. pestis* is centrifuged onto J774 or HeLa cells and then incubated 4 hours, after which the YopM or YopE distribution is determined by fluorescent antibody as in protocol 1) or by fractionating the culture and carrying out immunoblot analysis. Detailed examples of these protocols are given below.

#### Protocol 1: treatment of cultured cells with pure YopM

1.  $10^5$  J774 or HeLa cells are seeded into each chamber of a Labtek (Nunc) 4-chamber microscope tissue culture slide or onto 13 mm circular coverslips in a 24-well cluster dish (Corning).
2. Culture 24 h in RPMI 1640 (RPMI; Gibco) + 10% heat-inactivated (HI) fetal bovine serum (FBS) (Gibco) at 37°C with 5% CO<sub>2</sub>. (37/CO<sub>2</sub>)
3. If cytochalasin D or colchicine is being used (to poison processes dependent upon microfilaments or microtubules, respectively), they are added at 10 µg/ml (we tested 1, 5, and 10 µg/ml), and the cultures incubated 30 min at 37/CO<sub>2</sub>.
4. The medium is removed and replaced with the same medium containing YopM or YopM + cytochalasin D or colchicine. If YopM is to be visualized by a fluorescent antibody-based technique, it is added at 5 µM; if visualization is to be by immunoblot, 0.5 µM is used. Negative control samples get medium only.
5. Incubate 2 h, 37/CO<sub>2</sub>.
6. Wash 3X with 1-2 ml room-temperature (RT) PBS per chamber or well, leaving the PBS on for 5 min. (i.e., "5-min. changes"). In these protocols, PBS signifies solution "a" of Dulbecco's phosphate-buffered saline (containing 137mM NaCl, 2.7mM KCl, 8mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.5mM KH<sub>2</sub>PO<sub>4</sub> adjusted to pH 7.4 with NaOH; ref. 44).
7. If trypsin/EDTA is to be used to test surface-accessibility of

the YopM, it is added to give a final concentration of 0.05% trypsin, 0.53 mM EDTA; by diluting a 100X stock (Gibco), and the cultures are incubated 5 min, 37/CO<sub>2</sub>. Then wash the cultures 3 times as in step 6.

8'. If the analysis is to be by immunoblot, the cells are scraped, pelleted in a microfuge tube, and dissolved in SDS-PAGE sample buffer for subsequent standard SDS-PAGE and immunoblot analysis.

8. Withdraw PBS and fix 5 min. with -20°C methanol. (1 h in 2% paraformaldehyde, made in PBS and titrated to pH 7.4 resulted in dimmer immunofluorescence and was not tried again.)

9. Wash 2X as in step 6.

10. Block 30 min, RT with PBS containing 10% HI FBS + 1% mouse serum (to block Fc $\gamma$  receptors on J774 cells).

11. Replace blocking solution with 1° antibody (Ab). We use rabbit polyclonal anti-YopM IgG, diluted 1:1,000 in PBS (1:5,000 did not give detectable fluorescence). Control samples get only the buffer without 1° antibody. Incubate 1 h RT.

12. Wash 3X with PBS as in step 6.

13. Add 2° Ab (Goat anti-rabbit IgG conjugated to FITC; Sigma), diluted 1:160 in PBS and incubate 1 h RT in the dark.

14. Wash 2-3X with PBS.

15. Mount coverslips or add a coverslip to the Labtek slide after removing the chamber walls. We initially used a mounting medium we made ourselves (90% glycerol + 100  $\mu$ g/ml p-phenylene diamine) but now routinely use Vecta Shield (Vector, Burlingame, CA), in hopes that the samples will be less readily quenched by exposure to UV.

#### Summary of findings from protocol 1.

There were scattered fluorescent spots on both J774 and HeLa cells. These were absent if the trypsin-treatment was used, indicating the surface-localization of the fluorescing YopM. Confocal microscopy confirmed the surface-localization of the spots of YopM. Colchicine or cytochalasin D had no effect on the findings (not surprisingly, if YopM was not being taken up by the cells). The YopM used for these tests contained endotoxin, estimated by LAL assay to be 1  $\mu$ g/ml when YopM was present at 1 to 1.5 mg/ml (3 or 0.3 ng/ml when YopM was present at 5 and 0.5  $\mu$ g/ml) and should not have interfered with these assays by stimulating the J774 cells. Western analysis of total cell extracts revealed the same amount of YopM present for cells

treated with boiled or unboiled YopM.

These findings suggest that YopM aggregates might be sticking nonspecifically to both cell types, by virtue of the "spot" nature of the fluorescence and its retention at the cell surface. We will not continue these experiments with J774 or HeLa cells; however, we should test the ability of platelets to bind YopM, as somehow YopM inhibits thrombin-induced platelet aggregation even though it does not bind thrombin very strongly. YopM might bind specifically and tightly to platelets, as boiled YopM did not inhibit platelet aggregation but YopM did (2,3).

#### Protocol 2 -- infection of cultured cells with *Y. pestis*

1. The day before the experiment, initiate growth of the yersiniae. The final resuspension of the bacteria for adding to eucaryotic cells is in RPMI 1640 + HI FBS (10% for immunofluorescence analysis, 1% for immunoblots.) We have tried three pregrowth conditions and have not yet settled on a final choice:

a) growth in the defined medium TMH containing 2.5 mM  $\text{CaCl}_2$  (43) at 26°C overnight, dilution in this medium to  $\text{OD}_{620}$  of 0.2 and reinitiating growth, shifting the temperature to 37°C and incubating the rest of the day and overnight, diluting to  $\text{OD}_{620}$  of 0.5 in TMH +  $\text{CaCl}_2$  and incubating at 37°C 1 to 2 h before diluting 1:100 to 1:200 into RPMI 1640 + HI FBS (to give ca.  $2.5 \times 10^6$  cells/ml), and incubation by standing in 37/ $\text{CO}_2$  ca. 15 min. prior to adding to monolayers (to give MOI of 20 to 30);

b) growth overnight at 26°C in Heart Infusion Broth (HIB; Difco), diluting the next morning to  $\text{OD}_{620}$  of 0.2 in RPMI 1640 containing HI FBS, and incubating standing in 37/ $\text{CO}_2$  for 2 h prior to dilution in RPMI + HI FBS to give an MOI of 20 when added to monolayers;

c) growth overnight at 26°C in TMH + 2.5 mM  $\text{CaCl}_2$ , dilution to  $\text{OD}_{620}$  0.2 to 0.3 and incubation 1.5 h to reinitiate growth, shifting the temperature to 37°C and incubating 2 h more, and diluting into RPMI + HI FBS just before adding to monolayers (at MOI ca. 20).

2. Seed J774 or HeLa cells 1 or 2 days before the experiment:

For fluorescent antibody methods:

- a)  $10^5$  cells per chamber of the LabTek chamber slides.
- b)  $5 \times 10^5$  to  $10^6$  cells per 35 mm Petri dish containing 18 mm square coverslips.

For extracts and immunoblot analysis:  $5 \times 10^5$  to  $10^6$  cells per 35 mm Petri dish containing 18 mm square coverslips.

Culture these in RPMI + 10% HI FBS at 37/CO<sub>2</sub>.

3. Remove the medium from the cells and replace with 1 ml medium containing yersiniae (per chamber of chamber slides) or 2 ml per petri dish.

4. Centrifuge 5 min. at room temperature, 1,000 rpm, in Beckman TJ-6 swinging bucket centrifuge.

5. a) Incubate 4 h 37/CO<sub>2</sub>.

b) Incubate 30 min. 37/CO<sub>2</sub>; wash 3X with PBS; then incubate 4 h 37/CO<sub>2</sub>.

6. Remove medium (save this if doing immunoblots) and wash 2 to 3X with PBS (add the washes to the previously removed medium if doing immunoblots).

7. Immunofluorescence assays

a. To stain eucaryotic cells, incubate 5 to 10 min., RT, in 25 µg/ml Wheat germ agglutinin conjugated to Texas Red in PBS (WGA-TR; Molecular Probes, Eugene, OR).

b. Wash 2X with PBS.

c. Fix 5 min. with ice-cold methanol (2% paraformaldehyde made in PBS and titrated to pH 7.4 and used 1 h also works).

d. Wash 2X PBS

e. Block 30 min, RT with PBS containing 10% HI FBS + 1% mouse serum (to block Fcγ receptors on J774 cells). The mouse serum is not necessary for HeLa cells.

f. Replace blocking solution with 1° antibody (Ab). We use rabbit polyclonal anti-YopM IgG, diluted 1:1,000 in PBS + 1% mouse serum (1:5,000 did not give detectable fluorescence). Control samples get only the buffer without antibody. Incubate 1 h.

Other 1° antibodies we have used are a polyclonal rabbit anti-Yersinia antiserum we made previously, used at a dilution of 1:1,000, to visualize the yersiniae, and mouse anti-YopM (described previously) or mouse anti-YopE from Art Friedlander's group, both used at 1:1,000.

g. Wash 3X with PBS.

h. Add 2° Ab (Goat anti-rabbit IgG conjugated to FITC; Sigma), diluted 1:160 in PBS + 1% mouse serum.

Other 2° antibodies we have used were coupled to Texas Red or the blue fluorochrome AMCA (Molecular Probes). For a few tests with mouse antibodies as the 1° antibody, we used Goat anti-mouse antibodies coupled to FITC or Texas Red, also



from Molecular Probes. These tests failed to give detectable fluorescence, perhaps because the primary antibodies were not as high-titer as the rabbit antiserum.

i. Wash 2-3X with PBS.

j. Mount coverslips or add a coverslip to the Labtek slide after removing the chamber walls, using Vecta Shield.

#### 7. Immunoblot analysis

a. Lyse the eucaryotic cells:

- scrape cells in 1 ml PBS and transfer to a microfuge tube; add Triton-X-100 to give 1% and mix well;

or

- add 1 ml ice-cold water; incubate on ice 5 min and disrupt cells by vigorous pipetting. Transfer to a microfuge tube.

b. Pellet bacteria and other debris in microfuge at 14,000 rpm 25 min. Pellet can be suspended in SDS-PAGE sample buffer (e.g., 50  $\mu$ l) and analyzed if desired.

c. TCA precipitate the supernatant = cytosol overnight with 10% TCA, on ice. Microfuge 30 min at 4°C and discard supernatant. Neutralize pelleted proteins with 1  $\mu$ l of 1 M NaOH (checking by dotting  $\leq$  0.5  $\mu$ l onto pH paper). Suspend in 75  $\mu$ l SDS-PAGE sample buffer.

d. The medium plus washes saved from step 6 are either centrifuged or filtered to remove bacteria and then TCA-precipitated and the pelleted proteins neutralized as for cytosol samples; resuspend in 150  $\mu$ l SDS-PAGE sample buffer.

#### Summary of findings from protocol 2

Immunoblot Immunoblots show that YopM and YopE are partitioned ca. 50:50 between the cytosol and the eucaryotic cell (we have not yet analyzed the debris that would contain the bacteria). Control tests probing the samples with antibody against the yersinial cytoplasmic protein LcrH (which should be absent from eucaryotic cytosol preparations not containing contaminating yersiniae) indicate that there is negligible or no contamination of the cytosol with *Y. pestis* and hence that the Yops we see in this fraction represent Yops delivered into the eucaryotic cell by the vectorial translocation mechanism. We obtain the same findings whether J774 or HeLa cells are used. Because *Y. pestis* invades HeLa cells very inefficiently, this means that the delivery of Yops into the cells is occurring by extracellular *Y. pestis*, as is hypothesized. The relatively large amount of YopE in the culture medium was seen both when we infected and left all bacteria there for 4 h and when we removed any nonadherent yersiniae after 30 min and then incubated for 4 h. It also was seen when we tested a serotype 1 *Y. pseudotuberculosis* strain in

these assays. Unfortunately, *Y. pestis* appears to secrete some Yops when incubated alone in all tissue culture media so far tried (RPMI, Eagle's DMEM, Leibovitz L-15). Having YadA present in the *Y. pestis* strain has not significantly affected the results. However, we have some hope that we can get more efficient partitioning of Yops into eucaryotic cells, as one experiment done in a concurrently running project in my lab did find that the majority of YopM and YopE were targeted into the eucaryotic cell. In this instance, the bacteria were grown at 26°C in HIB (and were not allowed to enter stationary phase) and then were diluted into tissue culture medium (RPMI, DMEM, or Leibovitz L-15), allowed to stand at 37°C/CO<sub>2</sub> for 2 h prior to being added to cultured cells. Accordingly, we will build on this protocol in the future. Our experience shows that the efficiency of targeting Yops into eucaryotic cells is very condition-dependent, and we suspect that it may not be 100% even in vivo. The goal for our future experiments is to obtain efficient enough Yops translocation that we can obtain information about YopM's likely intracellular target.

Immunofluorescence. We could detect YopM in association with adherent extracellular yersiniae, but have not yet seen YopM within the cytosol of eucaryotic cells. The only exception might be one experiment in which we saw diffuse staining for YopM within vacuoles containing intracellular bacteria, but that was a rare observation. Typically, we did not detect YopM associated with intracellular bacteria or extracellular nonadherent yersiniae. We also have not seen YopE within the eucaryotic cytosol, and this Yop is known to be vectorially transferred into the cytosol and to assume a perinuclear distribution (35). Our failure to see intracellular YopM and YopE might be due to loss of a significant amount of these proteins to the medium, coupled with our use of a relatively weak antibody for detecting YopE: when *Y. enterocolitica* mutants did not tightly couple Yop secretion to vectorial translocation, very low intracellular amounts of Yops were seen (14). We anticipate that YopM is vectorially transported, because of our findings with immunoblots and because a YopM-CyaA hybrid protein is translocated into eucaryotic cells at a low efficiency (14). However, we do not yet know the efficiency of this process for native YopM or the final destination that YopM reaches. We are testing variations on the protocols for bacterial pregrowth and infection of eucaryotic cells to see if we can obtain a stronger immunofluorescence signal.

## Conclusions

### Aim 1

1. Based on tests with 3 human plague convalescent sera, YopM is expressed during plague in humans and elicits an antibody response. At present, we do not know if this phenomenon might be

diagnostically useful.

2. YopM is not a protective antigen for inbred or outbred mice immunized passively with either mouse or rabbit anti-YopM antibody.

3. YopM is not a protective antigen for inbred or outbred mice immunized actively with pure YopM.

4. Based on the above, YopM is not a good candidate for a subunit vaccine designed to protect via the humoral immune response. We do not know if YopM elicits a cytotoxic T cell response during a plague infection or if that is protective. If so, then designing a YopM vaccine component that elicits this protective response might offer a valuable component to an improved plague vaccine. During the final year of our Collaborative Agreement, we will finish the work planned under TO 2 by determining if there is a significant cytotoxic T cell component to the primary and secondary response to *Y. pestis* in mice, and we will endeavor to determine if this response includes YopM. This project will not further assess this issue. At this writing, Aim 1 otherwise has been accomplished.

5. YopM given intravenously is able to partially restore virulence to a YopM<sup>-</sup> *Y. pestis*, but it had no effect on lethality due to *Listeria monocytogenes*. This indicates that YopM can function from an extracellular location in a *Yersinia*-specific way, perhaps in conjunction with another *Yersinia* component. This finding of an effect of pure YopM on a *Yersinia* infection provides a clue about one potential function of YopM. During the final year of this project, we will make some tests to rule on likely ways that YopM may be acting extracellularly, as it is within the goal of the Collaborative Agreement to learn how YopM functions.

6. However, because YopM is not protective and likely is not accessible to antibody during its main mode of action in vivo, and because YopM interacts with thrombin only relatively weakly, we believe that YopM's main function is intracellular, and our efforts will be mainly focussed in learning how it might function intracellularly (the studies proposed for Aim 3).

#### Aim 2

7. We made a series of genetic constructs that encode YopM proteins having various regions deleted. This showed that the last 16 residues and LRRs 4 and 5 of YopM are not involved in binding thrombin, but that deletions that destroy YopM's putative leucine zipper or other structure provided by LRRs 7 and 8 abolish thrombin-binding. This work of analyzing our mutant YopM proteins is not complete: during the coming year, we will determine by sequencing the DNA of the constructs exactly what

mutations have been made and will analyze thrombin-binding by several mutant YopMs that have not yet been examined.

8. Thrombin's cleavage of YopM is independent of YopM's stable binding of thrombin at *abeI*: cleavage can occur in the absence of binding (as detected by obtaining a crosslinked YopM-thrombin product).

9. Our mutant *yopM* constructs provide a means to test whether thrombin-binding is a significant virulence function of YopM. During the next year, we will move several of these mutations into the virulence plasmid of *Y. pestis* and test the virulence of the resulting mutants. If a *Y. pestis* strain that expresses a YopM protein that does not bind thrombin is fully virulent or not as attenuated as a YopM<sup>-</sup> strain, then thrombin-binding is not a significant function of YopM in the pathogenesis of plague.

### Aim 3

10. We have shown that YopM is translocated into J774 macrophage-like cells and HeLa cells by *Y. pestis* brought into contact with the cells by brief centrifugation. This confirms an in-press report based on a YopM-CyaA fusion protein that some of YopM is vectorially translocated by adherent yersiniae into eucaryotic cells.

11. However, we get only ca. 50% of YopM going into the eucaryotic cell. This looseness of the control over vectorial translocation may depend on the conditions in which the bacteria are pregrown and incubated with the cells. We have not been able to visualize YopM's intracellular location in eucaryotic cells by immunofluorescence, probably because not enough YopM gets targeted into the cell to be detected by that method. During the coming year, we will vary the experimental conditions with the goal of being able to detect YopM targeting by immunofluorescence, as YopM's intracellular location will provide a clue about how YopM may be functioning.

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Annual Report for Collaborative Agreement DAMD17-94-C-4041  
Year 1: September 1, 1994-August 31, 1995

### Introduction

This project is evaluating the *Yersinia pestis* (plague) virulence protein YopM as a prospective component of a subunit plague vaccine. YopM is one of a set of proteins that are secreted into the culture medium by *Y. pestis* under conditions that are believed to mimic the contact of the bacteria with a phagocytic cell (1). Two of these proteins are directly targeted from the bacterium into the phagocyte, with resulting paralysis of phagocytosis. The fate of YopM is not known and will be investigated in this project (Aim 3). It is thought that at least some of YopM is likely to be present in the extracellular surroundings in tissues, because we have shown that YopM binds human  $\alpha$ -thrombin sufficiently tightly to prevent activation of platelets (2,3), and  $\alpha$ -thrombin is generated extracellularly by the activation of the serum protein prothrombin (4). YopM does not bind prothrombin (3); hence YopM is believed to act by sequestering thrombin as it is generated from prothrombin at foci of infection (1,3). This would be expected to have an anti-inflammatory effect, which could contribute to the virulence of *Y. pestis*, and indeed we found that YopM is necessary for the full virulence of *Y. pestis* in mice (2). Because YopM is thought to be located extracellularly, where it would be accessible to antibody, and is necessary for full virulence of *Y. pestis*, its activity might be neutralizable by antibody, thus affording some protection against plague. This is the basic hypothesis that is being tested in the Collaborative Agreement DAMD17-94-C-4041.

The three Aims in the Collaborative Agreement will examine different aspects of the hypothesis. The studies of Aim 1 will generate reagents (pure YopM and antibody against YopM) that will be used in all aims and will use these reagents to characterize the immune response to YopM and to test whether mice that are actively or passively immunized against YopM are protected against plague. These studies will directly ask the question of whether YopM is a protective antigen. Aim 2 will determine if thrombin-binding is the main function of YopM during an infection. We will make a *Y. pestis* KIM strain that expresses a mutant YopM that is defective in binding thrombin. If this *Y. pestis* strain is as avirulent as a strain that expresses no YopM at all, then thrombin-binding is YopM's main function; if not, then the existence of additional activities of YopM will have been revealed. In Aim 3, we will test the assumption that YopM is extracellular by determining whether YopM is secreted to the medium or into phagocytes or both when *Y. pestis* contacts a macrophage. If all of YopM is secreted into the medium, then the extracellular nature of YopM will be supported. If some or all of YopM enters macrophages, then we will have learned that YopM likely has a function other than, or in addition to, thrombin-binding. The studies of Aims 2 and 3 will better characterize

the mechanism of action of YopM, and the findings may be useful when considering potential toxicity of YopM to vaccinees.

### Body

Aim 1. Assess the immunogenicity and protective capacity of YopM

Technical objective (TO) 1. Determine if anti-YopM antibodies provide significant protection against lethal *Y. pestis* challenge.

Before the studies of this or the other Aims could be done, we needed to generate two reagents, pure YopM and a high-titer, YopM-specific antibody preparation. This reagent generation constitutes the first part of TO 1.

Purification of YopM We scaled up the method we developed previously (ref 3; summarized in Fig. 1) for purifying YopM. Our goal for this work was to have 1 band on a silver-stained gel and 1 band on an immunoblot probed with an existing rabbit antiserum directed against secreted proteins of *Y. pestis*.

### YopM Purification

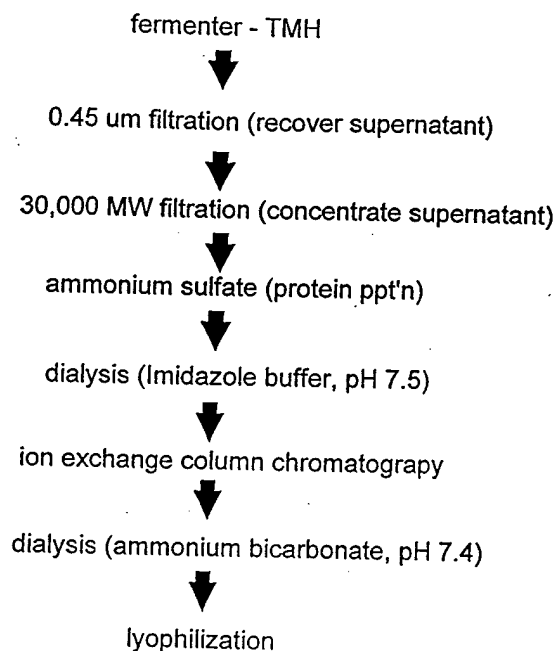
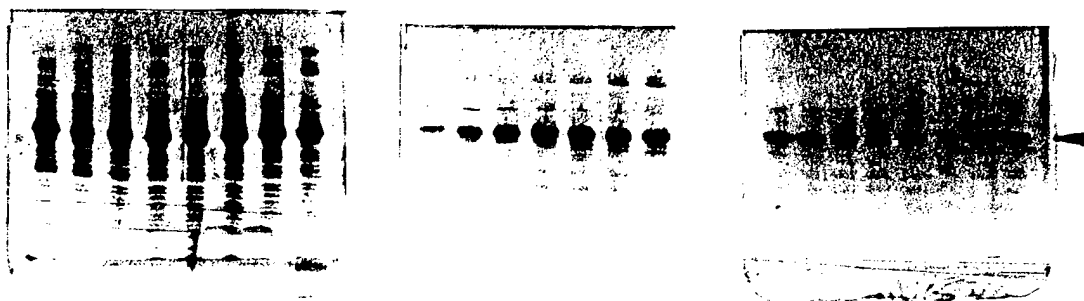


Figure 1. General flow of YopM purification protocol.

*Y. pseudotuberculosis* lacking its own *yopM* gene and that overexpresses the *Y. pestis* YopM is grown in the defined medium

TMH at 26°C as previously described (3), except that the concentration of potassium gluconate is increased 10-fold over that in conventional TMH to give 100 mM. We found that this helps delay the alkaline pH shift that occurs upon prolonged incubation at high density. Also, it is known from the early literature that availability of a fermentable carbohydrate equivalent to glucose supports the strongest expression of the secreted virulence proteins (5). Bacteria grown in flasks provide the inoculum for a 10-L culture in a New Brunswick magnaferm fermenter aerated at 14 L/min. and stirred at 400 rpm. The starting OD<sub>620</sub> typically is 0.3 to 0.4, and growth is continued at 26°C until the OD<sub>620</sub> reaches 0.8 to 1.5, at which point the temperature is shifted to 37°C to induce expression of YopM. The incubation is continued for as much as 18 h longer, with hourly checks of OD<sub>620</sub> and pH. As necessary, the pH is adjusted manually to 7.4 with glacial acetic acid, and foam is controlled by a mechanical foambreaker and addition as necessary of sterile Antifoam B emulsion (Sigma Chemical Co.). The cells are removed from the culture medium by passage over a 5 m<sup>2</sup>, tangential-flow, 0.45 µm pore-size, microporous membrane cassette in a Millipore Pellicon concentrator. The cell-free filtrate is concentrated to ca. 500 ml using a Millipore Minitan tangential-flow concentrator fit with a 30,000 Da cut-off ultrafilter. Proteins in the concentrate are precipitated by addition of solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to give 80% saturation and gentle stirring overnight at 4°C. Precipitated proteins are recovered by centrifugation, resuspended in imidazole buffer (0.05 M imidazole, 0.1% β-mercaptoethanol and 1 mM EDTA, pH 7.5 [3]), and dialyzed against imidazole buffer. This results in a 90-100 ml sample containing as much as 5 mg protein/ml. This sample is subjected to anion exchange chromatography at 4°C in a column containing 1.5 L of DEAE Sephacel resin (Pharmacia). After elution of nonbound and lightly bound proteins with 0.2 M NaCl, YopM elutes as a broad peak in a gradient of 0.2 to 0.5 M NaCl, with the peak of YopM occurring at ca. 0.35M NaCl. Fractions are collected as long as YopM keeps eluting and are pooled according to their heterogeneity in immunoblot analysis probed with a previously made rabbit anti-YopM antiserum (see below). The pools are dialyzed against 10 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.0, lyophilized, and stored at -20°C. We estimate that after 4 fermentations we generated a total of ca. 1.7 g of YopM.

Purity of YopM Fig. 2 shows silver-stained SDS-PAGE gels containing (A) peak fractions, (B) shoulder fractions, and (C) trailing fractions of YopM from DEAE chromatography.



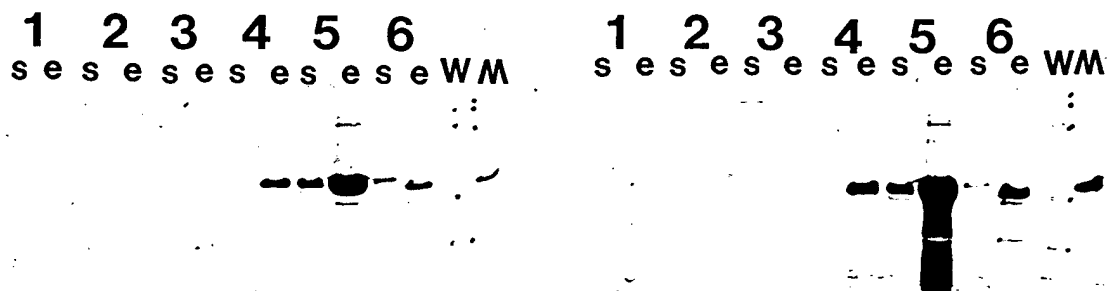
**Figure 2. SDS-PAGE analysis of sequential YopM-containing fractions eluted by a salt gradient from DEAE Sephacel. The leftmost gel shows the peak YopM fractions (numbers 38-45 of this column run); The central and right gels show shoulder and trailing fractions (numbers 46-52 and 53-60, respectively). The arrowhead indicates the 42 kDa YopM band.**

The peak fractions clearly appear to be very heterogeneous, with many YopM-cross-reactive bands smaller than the ca. 42 kDa main band of YopM, and a few larger species. In total, the extra bands constitute ca. 10% of the protein and are not seen in a Coomassie-stained gel unless many micrograms of YopM are loaded in the lane. The shoulder and trailing fractions appear to be "clean" YopM, by the criterion of 1 band on a silver-stained gel. We were distressed that the richest fractions appeared to be contaminated or degraded and devoted considerable effort at determining the source of the extra bands, in hopes of eliminating them.

The peak fractions from DEAE chromatography were subjected to fractionation by centrifugation over a Centricon-30 (Amicon Corp.) 30,000 MW cut-off filter, gel filtration on superose-12 (HPLC) or sepharose CL-6B (conventional chromatography), or hydrophobic interaction chromatography on butyl-sepharose developed with a 1 M to 0 M gradient of  $(\text{NH}_4)\text{SO}_4$ . (YopM sticks tightly in hydrophobic interaction chromatography and is not eluted at all from phenyl sepharose by this  $(\text{NH}_4)\text{SO}_4$  gradient. This suggests that YopM has hydrophobic patches that are exposed or become exposed during adsorption/desorption events in chromatography.) None of these procedures removed the extra bands seen after SDS-PAGE.

The extra bands were determined in a series of tests to be derived from YopM. In the experiment illustrated in Fig. 3, we prepared immunoblots in which YopM was detected using anti-YopM antisera that we raised previously (3): one raised against a peptide derived from the 12 C-terminal residues (356-367) of

YopM, and one raised against a sequence present twice at ca. the center of YopM (3,7). Figure 3 shows that both of these antisera are highly specific for YopM and show no significant reactions with proteins from the soluble cellular fraction (cytoplasmic plus periplasmic) or extracellular fraction (proteins released into the culture medium) of several *Yersinia* strains lacking YopM, including the *Y. pseudotuberculosis* host used in our work for overexpression of YopM (lanes 1-3). The only protein recognized in either the soluble or extracellular protein fractions of any of the YopM+ bacteria is YopM (lanes 4-6).



**Figure 3.** Immunoblot analysis of YopM-containing or lacking *Yersinia* fractions with anti-YopM peptide antibodies. The blot on the left is probed with antibodies derived against the YopM 12 C-terminal residues; the righthand gel is probed with antibodies against a 12-amino acid sequence that is present twice at ca. the center of YopM. Abbreviations: s, soluble cellular fraction (cytoplasm + periplasm); e, extracellular protein fraction (cell-free supernatant); W, prestained molecular weight markers; M, YopM from a peak fraction. Lanes: 1, YopM- *Y. pseudotuberculosis* strain 43 (background strain for overexpression of YopM; ref. 3); 2, *Y. pestis* KIM6 (lacking its Lcr virulence plasmid; YopM-); 3, *Y. pestis* KIM5 containing its Lcr virulence plasmid but having the *yopM* gene deleted (YopM-; ref. 2); 4, *Y. pestis* KIM5 YopH-mutant (YopM+; ref. 3); 5, *Y. pseudotuberculosis* 43 YopM overexpression strain (YopM++; ref. 3); 6, *Y. pestis* KIM5 (YopM+; ref. 2).

The multiple YopM bands are visualized with the internally-reactive antibody (Fig. 3 right), whereas mainly full-length YopM and some minor higher-molecular-weight forms are seen with the C-terminally reactive antibody (Fig. 3 left). This indicates that the smaller bands represent YopM from which various amounts have been removed from the C-terminus. The minor higher-molecular weight species could represent various aggregates of both processed and unprocessed YopM. The latter, containing at least one intact C-terminus, would be recognizable by the C-terminally reactive antibody. In other tests, when we subjected the peak-fraction YopM to chemical cross-linking, the smaller bands

disappeared: only monomer (42 kDa) and traces of higher molecular weight forms were seen in gels prepared by SDS-PAGE followed by silver staining (not illustrated). This indicates that in the native material of peak fractions, the smaller bands are closely associated with their C-termini so that these can be crosslinked to give a normal-sized YopM upon denaturation with SDS. This explains why no native chromatographic procedure was successful in eliminating the smaller bands of YopM from peak fractions: some of the YopM in these fractions is present as a full-sized but nicked protein, and the pieces stay together until they are dissociated by boiling in 2.3% SDS + 5%  $\beta$ -mercaptoethanol for SDS-PAGE analysis.

The *Yersinia* for the experiment of Fig. 3 were grown in conventional batch cultures harvested at OD<sub>620</sub> of ca. 0.8. at 7 h after shifting the temperature to 37°C. The multiple bands are present in the secreted YopM of any *Yersinia* strain expressing YopM but are most easily seen when more YopM is present (in the YopM-overexpressing *Y. pseudotuberculosis*), as this permits enough of each band to be present in the lane to be detectable. This and similar experiments not illustrated show that the degradation is not an artifact of the fermentation process or of subsequent handling: it is detectable as soon as YopM is present in useful amounts in the culture medium. Addition of a serine protease inhibitor (Pefabloc SC from Boehringer Mannheim) to a culture at the time of the temperature shift or just prior to harvest was not helpful. The latter (before harvest) had no effect, and the former generally inhibited expression and secretion of Yops. It is not feasible to add metalloprotease inhibitors such as EDTA, as a high Mg<sup>2+</sup> concentration is needed to get strong expression of the secreted *Yersinia* virulence proteins (5,6). A storage test at 37°C overnight showed that YopM by itself does not autoproteolyse.

In another test to characterize our YopM fractions, (Fig. 4), the shoulder/trailing fractions gave one band in an immunoblot probed with an antiserum raised against a preparation of total extracellular (secreted) *Yersinia* proteins (Fig. 4 left), and the peak fraction gave the same pattern it gives when probed with the YopM-specific anti-internal YopM peptide antibody (compare Fig. 4 left to Fig. 4 right). These findings show that both peak and shoulder/trailing fractions are not detectably contaminated with other extracellular proteins.

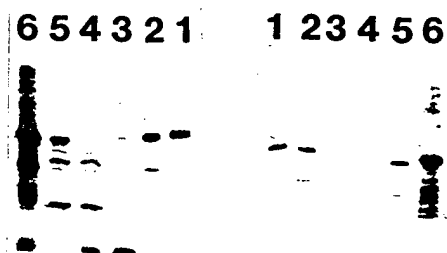


Figure 4. YopM is not contaminated by other extracellular proteins. YopM peak (lanes 2) or trailing (lanes 1) fractions were probed in immunoblots with an antiserum directed against total *Yersinia pestis* extracellular proteins (left) or anti-YopM peptide antibody directed against an internal epitope (right). Other lanes contain crude preparations of secreted proteins: 3, the *Y. pseudotuberculosis* strain 43 background strain (YopM-); 4, YopM- *Y. pestis* KIM5 mutant; *Y. pestis* KIM5 (YopM+); 6, YopM expression *Y. pseudotuberculosis* 43 strain (YopM++).

There is one final attempt we are making to see if we can get a greater amount of un-nicked YopM. It is not known if YopM has a specific Yop chaperone (Syc) that might help protect it from degradation (8). If it does, and if the Syc is encoded by DNA adjacent to the *yopM* gene as with other Syc-Yop pairs, then the *yopM* clone in the expression construct might not contain the *syc* gene. The *Y. pestis* virulence plasmid in the *Y. pseudotuberculosis* YopM overexpression host might express enough of the Syc to permit secretion of the YopM encoded by both the virulence plasmid and the overexpression clone that are present in this strain (3), but the Syc might be limiting for protection of all of the YopM against nicking by proteases. To test this idea, we will create a different expression construct, which based on our previous work (3) should still overexpress YopM but which now will have flanking DNA that contains an ORF (labeled ORF 15.9 in Fig. 6 of ref. 3) that could potentially encode the hypothetical SycM. We will determine if the YopM secreted by this strain is more intact than that produced by our present expression strain.

Bottom line on purity. Both the peak and shoulder or trailing fractions are pure YopM, by the tests shown in Figures 3 and 4. Apparently, some of the YopM is C-terminally processed to various extents by proteases in or on the bacteria, resulting in a broad elution profile on anion exchange chromatography. The peak YopM fractions mainly contain full-length YopM (judging by reactivity with the anti-C terminal antibody), but some nicked molecules are present (and seen in heavily loaded gels treated to silver staining or to immunoblotting probed with anti-internal

peptide antibody), and these have retained their nicked fragments during chromatography. The nicking is revealed by SDS-PAGE, when the protein fragments are separated after boiling in SDS. We believe that all of these pure YopM fractions are suitable for immunization of mice, as the mice will process the protein anyway (and the nicked form is only about 10% of the total). For the functional studies of Aims 2 and 3, only the most intact material should be used (of which we have the least amount, compared to the amount from peak fractions). For the work to be described below, we actually only used the shoulder/trailing fractions. On 3/23/95, we sent to Col. Art Friedlander at USAMRIID 100 µg of trailing YopM fraction and samples of our previously made rabbit anti-peptide antisera directed against the YopM C-terminus and the internal YopM epitope. As a deliverable part of the Collaborative Agreement, we will send ca. 5 mg of pure YopM to USAMRIID after we can remove endotoxin and provide useful information from our other studies in Aim 1.

Aggregation state of YopM. During our efforts to eliminate the multiple bands of YopM seen in peak fractions, we found that on a superose-12 HPLC column developed with 0.01 M (NH<sub>4</sub>)HCO<sub>3</sub> buffer pH 8, YopM eluted with the same elution time as did catalase (232,000 Da) and that no YopM eluted when monomer should appear. However, upon subsequent SDS-PAGE, the YopM in the fractions recovered from the superose column was dissociated and was all of the monomeric size. This suggested the surprising possibility that native YopM is hexameric and becomes monomeric only when denatured. As this would affect our models for how YopM interacts with thrombin, we needed to confirm this finding or identify conditions in which YopM does or does not oligomerize, so that its aggregation state could be controlled. We conducted a series of superose-12 column runs with various buffers and native gel electrophoresis runs with YopM in various buffers. We saw higher molecular weight forms become major components of the sample when the pH was above 8 and/or when YopM was subjected to gel filtration on superose-12:

a.) If the YopM was first dissolved in PBS (pH 7.4) or imidazole buffer (0.05 M imidazole + 1mM EDTA + 0.1% β-mercaptoethanol, pH 7.5) and then subjected to native electrophoresis, monomer was by far the major species. Traces of higher molecular weight species were present (bands of sizes that could represent dimer, trimer, tetramer, hexamer).

b.) Addition of 0.15 or 0.4 M NaCl to YopM in imidazole buffer prior to subjecting it to native gel electrophoresis had no effect (i.e., the YopM was predominantly monomeric).

c.) If YopM was dissolved in PBS and then subjected to gel filtration on superose-12 developed with PBS, the majority of YopM eluted as the trimeric size.



d.) If YopM was dissolved in the same pH 8.3 Tris Glycine used for native gels and then subjected to native electrophoresis, trimer was a major species, but the amount of monomer was still significant.

e.) If the YopM was dissolved in Tris Glycine native gel electrophoresis buffer and then subjected to gel filtration on superose-12 developed with this buffer, essentially all of the YopM eluted as the trimeric size.

Interestingly, the trimeric form obtained from gel filtration was mostly stable upon subsequent native gel electrophoresis: predominantly trimeric but also some monomeric YopM was obtained after the electrophoresis. The column fractions could be stored 2 to 3 days in the refrigerator and the native gel run with the same results. From these experiments, we learned some important things:

a.) YopM can oligomerize in a specific way. This was not just nonspecific aggregation, because we did not see forms larger than the hexameric size.

b.) High pH promotes oligomerization but salt does not.

c.) YopM's interaction with the agarose-based resin used for gel filtration promotes oligomerization.

d.) The oligomers, once formed, are relatively stable. The possibility is raised that they could occur in vivo, when YopM interacts with other proteins or cells.

e.) PBS and imidazole buffer are conditions that minimize oligomer formation. We had previously demonstrated YopM's ability to sequester thrombin and prevent platelet aggregation using YopM in PBS (2,3). Hence, YopM interacts with thrombin as a monomer; but the oligomers also potentially can bind thrombin and in fact do (see later).

Removal of endotoxin from YopM. Prior to using YopM in mice, we need to remove contamination by endotoxin. We take as our criterion of purity that there should be less than 1 ng endotoxin in each dose to be injected into mice. By the standard *Limulus* amoebocyte lysate assay (kit from BioWhittaker), a 1 mg/ml solution of YopM contained 35 µg endotoxin/ml. A 40 µg dose of YopM would contain 1.4 µg endotoxin. We tested two products for endotoxin removal, Detoxi-Gel (Pierce) and End X-B52 (Cape Cod Associates). Both of these retain YopM as well as endotoxin. Our tests did not identify conditions that would permit elution of YopM from Detoxi-Gel while retaining endotoxin (summarized below). However, we found that if we included 0.1% deoxycholate (DOC) in the YopM sample and elution buffer, then after incubation with End X-B52 beads overnight at 4°C, YopM did not stick to the End X-B52, while endotoxin was retained by the beads. The DOC is then removed from YopM by dialysis. This

procedure permitted us to clean up enough YopM to within our purity criterion (of < 1 ng endotoxin per mouse per immunization) to proceed with the work of Aim 1.

Summary of trials for endotoxin removal that failed.

1. Gel filtration on sepharose CL-6B (0.01 M  $\text{NH}_4\text{HCO}_3$ , pH 8 buffer)
2. Detoxi-Gel used as recommended by the manufacturer (YopM in PBS). Both YopM and endotoxin stuck.
3. Detoxi-Gel with adding 100  $\mu\text{g/ml}$  heparin (Sigma) to YopM (in PBS) and washing with PBS + heparin: neither YopM nor endotoxin stuck.
4. Detoxi-Gel with adding 1% or 10% fetal bovine serum (FBS) to YopM in PBS and washing with 3 column-volumes of PBS + FBS. Both YopM and endotoxin stuck.
5. Detoxi-Gel with 0.1% DOC added to YopM solution (in PBS) and washing with several column volumes of PBS + 0.1% DOC: both endotoxin and YopM stuck.
6. End X-B15 (same product as End X-B52 but a smaller amount for smaller YopM samples) with 0.05% or 0.1% octylglucoside added to YopM in PBS and to the PBS solution used for column elution. Both YopM and endotoxin eluted from the column.

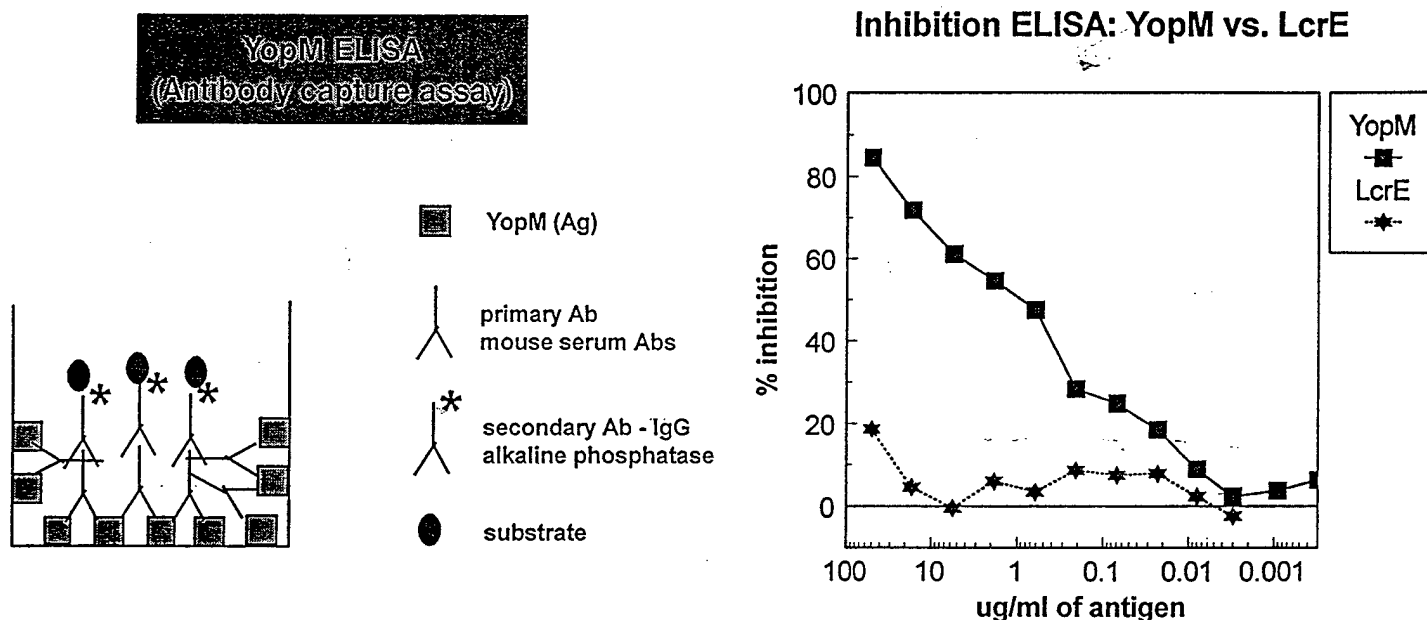
The use of End X-B52 is very expensive: it costs \$125 for the End X-B52 needed to clean up 5 mg of YopM, and the beads cannot be regenerated for reuse. We are exploring an alternative approach to get the cost down. Sartorius Q15 syringe filters (\$36 @) retain endotoxin. However, these do not yet perform satisfactorily for us: 1) YopM sticks, and has to be removed by using 0.1% DOC in the YopM sample and elution buffer; however more than half of the YopM stays on the column (i.e., is lost), and 2) the YopM that we do get off is not clean enough, although a great deal of endotoxin is removed from it. We presently do not know if the Q15 filter can be made to clean YopM adequately and with a good recovery of YopM, or what the loading capacity is of these filters for the first use, and we have not identified conditions that allow successful reuse. Although these filters can be regenerated with 0.2N NaOH, on subsequent uses they retain both YopM and endotoxin. Deoxycholate at concentrations from 0.2% to 1% does elute YopM from the regenerated filter, but some of the endotoxin also comes off. We will explore ways to remove endotoxin from YopM to the point that we can do the proposed studies within our budget (taking into consideration the cost of our time spent in optimizing cost and efficiency of endotoxin removal).

Anti-YopM antibody and control antibody from mice. For passive protection tests, we need anti-YopM antibody and control antibody raised in mice, as the tests will be done with mice. We initially chose to obtain the antibody from ascites as previously described (9,10). 40 12-13 week-old female Balb/c mice were immunized intraperitoneally (IP) with 0.2 ml of immunogen: 20 received a solution containing 40  $\mu\text{g}$  YopM in pyrogen-free

phosphate-buffered saline (PBS) emulsified 1:1 with Freund's complete adjuvant (FA), 10 received PBS:FA (1:1), 5 received 40 ug YopM mixed with Adjuvax (AV; from Alpha Beta Technology; AV powder was added to YopM in PBS to give a final concentration of 1 mg/ml and mixed by multiple draws through an 18 G needle on a syringe), and 5 received PBS:AV (prepared as for YopM:AV). After the serum anti-YopM concentrations reached peak levels, the mice were primed IP with 0.3 ml pristane (2,6,10,14-tetramethylpentadecane, Sigma). Ca. 1 to 2 weeks later, each mouse received IP  $2 \times 10^6$  murine XS63 myeloma cells obtained from ATCC). The exact treatment schedules were as follows:

Day	Immunization	Bleed	Pristane	Myeloma Cells
0-2		Prebleed		
2	40 $\mu$ g YopM or PBS			
15		Bleed #2		
16	40 $\mu$ g YopM or PBS			
36		Bleed #3		
37	40 $\mu$ g YopM or PBS			
45			FA mice	
49		Bleed #4		
52			AV mice	
60				All mice
73+	Collect ascites weekly			

We use a standard type of antibody capture assay (YopM ELISA) for analyzing sera for the presence of anti-YopM antibody (illustrated in Fig. 5 left and detailed below). Fig. 5 right shows data from an inhibition ELISA, in which either various amounts of pure YopM or pure irrelevant protein (LcrE, another *Y. pestis* virulence-related protein) are added along with the primary (anti-YopM) antibody in the YopM ELISA (protocol given below). The effective competition by YopM but not LcrE illustrates that the YopM ELISA is specific for YopM.



**Figure 5. Analysis of anti-YopM titer of mouse serum or ascites by YopM ELISA (left) and YopM Inhibition ELISA (right).**

Procedure for the YopM ELISA.

1. Coat a Nunc Maxisorp flat-bottomed 96-well plate overnight at 4°C with 4 µg/ml YopM in PBS (pH 7.4), using 100 µl per well.
2. Dump contents of plate and add 200 µl per well of 2% (wt/vol) powdered skim milk in PBS. Incubate overnight at 4°C.
3. Dump contents of plate and wash wells 3 times with ca. 250 µl PBS containing 0.05% (vol/vol) Tween-20 and 0.02% (wt/vol) NaN<sub>3</sub> (PBS-TA). Each change is incubated 3 min prior to dumping.
4. Invert and bang plate on absorbent paper to remove traces of liquid.
5. Add 100 µl primary antibody (dilutions of mouse serum) to wells, arranging for all dilutions to be tested in duplicate or triplicate wells. One column of wells to serve as blank gets 100 µl PBS-TA.

6. Incubate at room temperature 2 h.
7. Dump well contents and wash and drain as in steps 3 and 4.
8. Add 100  $\mu$ l secondary antibody (alkaline phosphatase-conjugated goat anti-mouse IgG [ $\gamma$  chain] (Sigma) at 1:20,000 in PBS-TA) to all wells including blanks.
9. Incubate at room temperature for 2 h.
10. Dump contents of wells and wash.
11. Add 100  $\mu$ l of pNPP substrate (1 mg/ml p-nitrophenylphosphate in 0.2 M Tris.HCl, pH 8.0; we use Sigma Fast tablet form of this reagent) to all wells.
12. Incubate at room temperature or 37°C for a pre-determined time that will be used for other ELISA tests to be compared to this one.
13. Stop the reactions by adding 25  $\mu$ l 3N NaOH to all wells.
14. Read OD<sub>405nm</sub>.
15. Average the readings for the duplicate or triplicate wells. The titer of a given sample is the dilution giving an OD reading equal to or greater than 2 times the standard deviation of the blank wells.

Procedure for the YopM Inhibition ELISA.

1. Coat Nunc Maxisorp 96-well plate with 100  $\mu$ l/well of a 4  $\mu$ g/ml solution of YopM in PBS (pH 7.4). Incubate overnight at 4°C. Then block overnight at 4°C with 200  $\mu$ l per well of 2% (wt/vol) powdered skim milk in PBS.
2. Prepare 12 serial 3-fold dilutions of YopM ranging from 100 to 0.0006  $\mu$ g/ml in PBS-TA, 230  $\mu$ l of each, in microfuge tubes. Into separate microfuge tubes, prepare 10 serial 3-fold dilutions, 230  $\mu$ l of each, of irrelevant antigen (LcrE in the experiment of Fig. 5) ranging from 100 to 0.006  $\mu$ g/ml. The 11<sup>th</sup> tube in this series will receive serum but no antigen (0% inhibition control); it receives 230  $\mu$ l PBS-TA now. The 12<sup>th</sup> tube receives only PBS-TA, 460  $\mu$ l (blank).
3. Prepare anti-YopM serum (for the experiment of Fig. 5 right, the serum was pooled from bleed #4 of mice immunized IP with YopM + FA):

Dilute serum as desired in PBS-TA (1:2500 for Fig. 5).

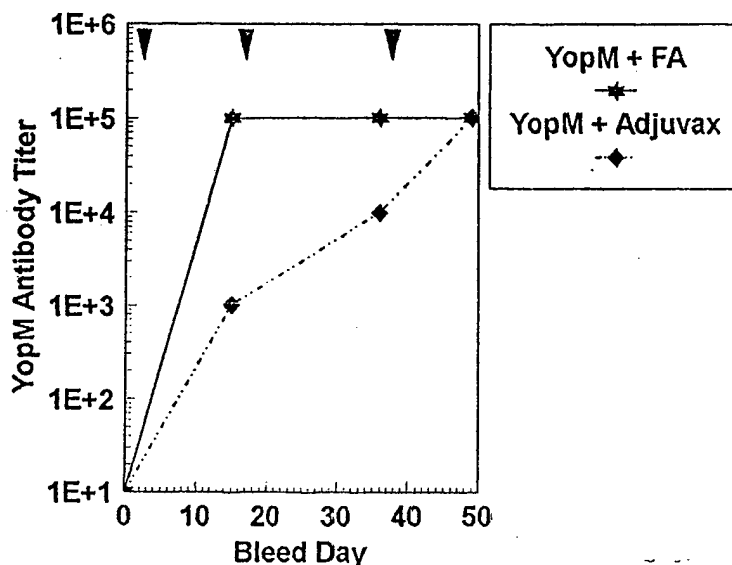
4. Add 230  $\mu$ l of serum to all tubes containing YopM and to tubes 1 through 11 in the irrelevant antigen series. The

final serum dilution = 1:5000 for the experiment of Fig. 5;  
the final antigen concentrations = 50 to 0.0003 µg/ml.

5. Incubate samples overnight at 4°C.
6. Dump skim milk solution from plate and wash as for normal ELISA (steps # 3 and 4 of YopM ELISA).
7. Add 100 µl of each sample from step 4 to each of duplicate wells in the Nunc plate.
8. Incubate at room temperature for 2 h.
9. Dump plate contents and wash.
10. Add 1:20,000 dilution of goat anti-mouse IgG (γ chain specific) alkaline phosphatase-conjugated Ab (Sigma) in PBS-TA to all wells (100 µl/well).
11. Incubate plate 2 h at room temperature.
12. Wash plate.
13. Add 100 µl/well substrate (Sigma Fast pNPP) and incubate 30 minutes at 37°C.
14. Measure the OD<sub>405nm</sub>, using wells containing sample from irrelevant antigen Tube #12 as blanks.
15. Determine average OD values for the duplicate wells.  
% inhibition =

$$\frac{\text{OD}_{405\text{nm}} \text{ 0\% inhibition control} - \text{OD}_{405\text{nm}} \text{ sample well}}{\text{OD}_{405\text{nm}} \text{ 0\% inhibition control}} \times 100\%$$

**Effect of adjuvant choice on anti-YopM IgG titer  
in sera from immunized mice**



**Figure 6. Effect of adjuvant choice on anti-YopM IgG titer in sera from immunized mice. Sera were pooled from mice for each bleed and titered by the YopM ELISA. Arrowheads indicate immunizations.**

Fig. 6 shows the timecourse for development of serum anti-YopM antibody in the immunization experiment described above. Mice immunized using FA reached their peak anti-YopM titer after a single immunization, by day 15. Mice receiving AV required significantly longer to reach their peak titer, but their titers did eventually match those of the FA-treated mice. To our dismay, the titer of anti-YopM in the ascites is 10-fold lower than that in the serum. This makes ascites collection only marginally better than blood as a source of antibody. Ascites induction prolongs the process of obtaining needed antibody, and the myeloma eventually kills the mice. To get enough antibody for the planned passive protection studies, we will need to use more mice and have begun immunizing 40 more mice: 20 receiving YopM:FA and 20 receiving PBS:FA. We will omit treatment with pristane and myeloma cells and collect blood weekly from the tail. The rest of the work in TO 1 (passive protection studies) will be begun when we have enough antibody.

**TO 2. Characterize the immune response in mice immunized with YopM**

**Immunization route and quality of humoral anti-YopM response.** Before carrying out active immunization experiments in which we will be challenging immunized mice with *Y. pestis*, we need to choose the best route of immunization. For this, we need to know the timecourse of induction of the antibody response for various possible routes of immunization, and whether different routes of

immunization elicit qualitatively significantly different antibody responses. To make these assessments, we immunized female, 8-12 week-old, Balb/c mice with 40 µg doses of YopM according to the schedule detailed below.

Day	Immunization	Bleed
0-1		Prebleed
2	#1	
15		Bleed #2
16	#2	
29		Bleed #3
33	#3	

Four routes of immunization were tested: IP in the groin area, using a 200 µl dose and a 20 G needle; subcutaneous at 2 sites on the back, using 100 µl doses and a 20 G needle (SC); intramuscular in the thigh, using a 50 µl dose and a 25 G needle (IM); and intradermal 2 sites near the base of the tail, using 25 µl doses and a 25 G needle (ID). There were 20 mice per group: 10 received YopM and 10 received PBS. For the IP and SC routes, the YopM doses contained YopM emulsified 1:1 with FA (hence the larger dose volume for those routes). No adjuvant is recommended for the IM and ID routes; also, these routes are not recommended for getting a good antibody response in mice, although they are good for humans (11).

**Table. Concentrations of antibody isotypes and subclasses in mouse serum after multiple immunizations with YopM of *Yersinia pestis*.**

Immunization Route	Serum Antibody Concentration (mg/ml)					
	IgA	IgG1	IgG2a	IgG2b	IgG3	IgM
Intraperitoneal <sup>a</sup>	0.013	11	0.15	0.9	0.15	0.022
Subcutaneous <sup>a</sup>	0.005	10	0.02	0.1	0.15	0.022
Intramuscular	0.0006	1.5	0.1	0.045	0.01	0.03
Intradermal	0.0006	0.2	0.006	0.01	0.01	0.02

<sup>a</sup> YopM administered with Freund's adjuvant.

The Table gives the concentrations of various Anti-YopM antibody isotypes and subclasses elicited in the experiment



described above. Data are given for Bleed #3. As expected (12), the predominant antibody type elicited is IgG1, and this was true for all routes tested. This also is the predominant serum antibody type in humans (12). It was not expected that there would be much IgM in hyperimmunized animals; nor did we anticipate that there would be a significant IgA titer, as a mucosal immune response would not have been significantly stimulated by any of the routes. IP and SC immunization produced the highest titers, probably because FA was used. At bleed #2, the anti-YopM titer for the SC route was 10-fold lower than for IP, but by bleed #3, the titers were comparable. We conclude that we should use either IP or SC immunization for experiments that aim to test whether active immunization with YopM can be protective. It is beyond the scope of this project to attempt to mimic in mice the exact route and adjuvant combination that might be used in humans: that is an issue that will arise once it is known whether YopM can be a protective antigen. It makes sense to use a more central (IP) than peripheral (SC) immunization route for our particular plague model, as we must challenge intravenously (IV) with *Y. pestis* KIM. We plan to focus our efforts next on TO 3, to make direct tests for protection by active immunization with YopM.

Aim 2. Determine if thrombin-binding is necessary for YopM function in vivo.

TO 1. Identify thrombin-binding sites on YopM.

The overall goal of this Aim is to improve our understanding of how YopM functions in tissues, so as to be informed about effects it might have on vaccinees. We know that YopM binds  $\alpha$ -thrombin (below, we interchangeably use "thrombin" and " $\alpha$ -thrombin"); we would like to know how it does this and what the likely consequences are on thrombin's many activities. We also want to know if thrombin-binding is the only or main activity of YopM. If not, then we are alerted to other potential effects of YopM when injected into people. These initial research goals boil down to 2 questions: Where on thrombin does YopM bind? and Where on YopM does thrombin bind? The answer to the second question will focus our efforts at site-directed mutagenesis to create a *Y. pestis* strain that makes YopM that is defective in thrombin-binding. We will then test that strain for its virulence in mice and determine quantitatively how significant YopM's thrombin-binding is in plague.

Where on thrombin does YopM bind?  $\alpha$ -Thrombin is a serine protease, with many structural similarities to trypsin but with a much greater specificity: although it cleaves after basic residues with a preference for R, it cleaves only 4 of the 376 R/K-Xaa bonds in fibrinogen (13-16). Its active site is in a deep pocket, partially occluded by two protruding loops that limit access of potential substrates. Moreover, thrombin's

activity on protein substrates (as opposed to small-molecule synthetic substrates) requires binding of the substrate at one or more region distinct from the catalytic site. The catalytic pocket is flanked to the "west" (in the standard "front" view of thrombin) by an apolar binding site and to the "east" by anion-binding exosite I (abeI, also called the fibrinogen recognition exosite). This latter is a positively charged surface patch with hydrophobic crevices.

Substrates (fibrinogen, thrombin receptor, coagulation factor VIII), most inhibitors (the leech anticoagulant hirudin, heparin cofactor II), and activity modulators (thrombomodulin) bind to abeI by means of linear peptide sequences having clusters of negatively charged residues interspersed with hydrophobic residues that insert into the hydrophobic crevices of abeI. There is no strict consensus sequence for this abeI-binding region of these molecules (13-17). Models for how these molecules bind to abeI are based on information from the crystal structures of thrombin bound to hirudin, to the sulfated dodecapeptide hirudin tail called hirugen, and to peptides derived from the thrombin receptor (13-17). Hirudin, a 65-residue peptide, has a disulfide-knotted head that associates with the apolar site on thrombin, a noncleavable K/P bond, and an anionic tail. The tail binds in an extended conformation to abeI. Hirudin or hirugen binds  $\alpha$ -thrombin with very high affinity ( $K_d = 10^{-13} - 10^{-14}$  M for hirudin) and competitively inhibits binding of thrombin's substrates and peptidic inhibitors. Our data (see below) indicate that abeI binds YopM. YopM must also be able to associate with the catalytic site, because it is inefficiently cleaved by thrombin. However, YopM's ability to inhibit activation of platelets by thrombin probably is mainly due to the blockage of abeI by YopM.

Chemical crosslinking assay. We have been using chemical crosslinking assays (3) to define YopM's binding site on thrombin, because these are already working in our hands, they do not require that the catalytic site of  $\alpha$ -thrombin be tightly masked by YopM, which is only inefficiently cleaved by thrombin (see below), and they do not require that all of thrombin in a reaction mixture be complexed with YopM at all times. In these assays, YopM and  $\alpha$ -thrombin (human  $\alpha$ -thrombin from Haematologic Technologies, Inc.) and competitor are mixed, usually in a 1:1:>1 molar ratio, in phosphate-saline buffer (PD) (soluton "a" of Dulbecco's phosphate-buffered saline [18]), giving 25  $\mu$ l final volume. In some experiments, YopM and  $\alpha$ -thrombin are preincubated for 20 min at room temperature before adding competitor. This permits some cleavage of YopM to occur and be revealed in the final immunoblot. In other configurations of the assay,  $\alpha$ -thrombin and competitor (in PD) might be preincubated before adding YopM; then there might be another preincubation of all components. After all preincubations, 1  $\mu$ l of 25-fold concentrated crosslinker is added to give a final concentration of 5 mM, and the crosslinking reaction is allowed to occur for 30 min. at room temperature. The reaction is stopped by adding 1 M

Tris.HCl, pH 8.0 to give a final concentration of 50 mM and incubating for 15 min. (room temperature). The reaction is analyzed by immunoblot analysis probed with anti-YopM or anti-thrombin antibodies.

We tested a panel of crosslinkers (all from Pierce) with different chemical targets, aqueous solubility, cleavability after crosslinking, and spacer arm length to be certain that we would be using the optimal system for these studies. A summary of those tests is given below.

Xlinker <sup>a</sup>	Target <sup>b</sup>	Solubility	Cleavable Arm (Å)	Results <sup>c</sup>
DSS	NH <sub>2</sub> /NH <sub>2</sub>	-	-	11.4 2+
BS <sup>3</sup>	NH <sub>2</sub> /NH <sub>2</sub>	+	-	11.4 +
DSG	NH <sub>2</sub> /NH <sub>2</sub>	-	-	7.7 2+
BMH	SH/SH	-	-	16.1 -
SMCC	SH/NH <sub>2</sub>	-	-	11.6 2+
Sulfo-EGS	NH <sub>2</sub> /NH <sub>2</sub>	+	+	16.1 ±

<sup>a</sup> BMH = bismaleimido-hexane, BS<sup>3</sup> = bis(sulfosuccinimidyl) suberate, DSG = disuccinimidyl glutarate, DSS = (disuccinimidyl) suberate, SMCC = succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, and Sulfo-EGS = sulfo-ethylene glycolbis-(succinimidylsuccinate).

<sup>b</sup> "Target" designates the reactive groups on YopM or thrombin that are attacked by the two ends of the crosslinker.

<sup>c</sup> Results designate the intensity the band due to the ca. 74 kDa crosslinked product believed to contain 1 molecule each of YopM and thrombin.

The best crosslinking was observed with aqueous-insoluble crosslinkers. We have chosen to continue our use of DSS, which was the crosslinker we first used (3). Other information we gained from this experiment will be discussed later.

Figure 7 decodes the identities of the various bands we see in the crosslinking assays. We set up the assays with 5-fold higher concentrations of reactants compared to those used in ref. 3 to enhance the intensities of the bands and thereby provide a convincing demonstration of effects of competitive inhibitors. The anti-YopM2 antibody (left lanes) reacts strongest with YopM, but crossreacts with thrombin; the anti-thrombin antibody (right lanes) is thrombin-specific.

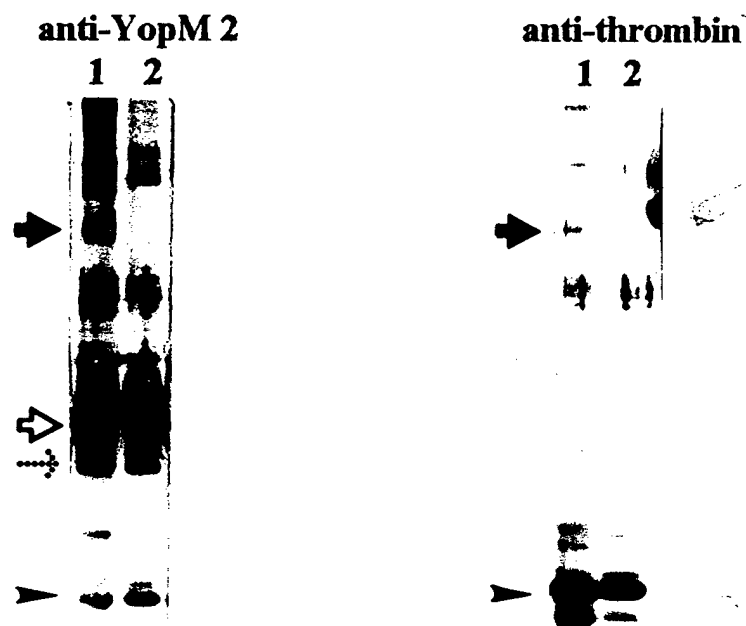


Figure 7. Hirudin inhibits formation of the YopM-thrombin crosslinked product. In lanes 1, YopM and  $\alpha$ -thrombin, each at 5  $\mu$ M, were preincubated before being crosslinked. In lanes 2, YopM and thrombin, each at 5  $\mu$ M, were mixed and preincubated, then 10  $\mu$ M hirudin was added, and the mixture was crosslinked. The samples were subjected to immunoblot analysis probed with antibody raised against the whole YopM molecule (anti-YopM2, left set of lanes) or antithrombin antibody (from American Diagnostica, right set of lanes). Solid arrows, 74 kDa crosslinked YopM-thrombin product; open arrow, 42 kDa YopM; dotted arrow, product of YopM cleavage by thrombin; solid arrowheads, 34 kDa  $\alpha$ -thrombin.

The features in lane 1 left are as follows (from low to high molecular mass):

1. Family of bands due to thrombin (arrowhead).
2. YopM degradation product(s) (main one indicated by dotted arrow). These are seen only when YopM is preincubated with thrombin as in this experiment (see

also Fig. 8, below).

3. YopM (open arrow). Note that the crosslinking does not eliminate either this band or that due to thrombin. Tests with higher concentrations of crosslinker (to 10 mM) gave similar results. We think that this indicates that there is a high rate of association/dissociation of YopM and thrombin such that only a portion is captured as a complex.
4. The family of weak bands between the open and closed arrows is variably seen (even when YopM is crosslinked alone, without thrombin present) from experiment to experiment.
5. Primary crosslinked product of YopM and  $\alpha$ -thrombin (solid arrow). This is weakly detected by anti-thrombin antibody. This suggests that epitopes of the anti-thrombin antibodies are at least partially obscured when YopM or crosslinker binds to thrombin.
6. Higher molecular weight bands. These represent YopM dimer (seen most clearly in lane 2, center), thrombin bound to YopM dimer, and higher aggregates of YopM with thrombin bound. Recall that small amounts of YopM dimer and higher aggregates are present to varying degrees in the YopM preparations (see earlier) and are stabilized by crosslinking (3).

Lanes 2 show that hirudin (Accurate Chemical and Scientific Corporation), present at a 2-fold molar excess over YopM or thrombin, can completely displace all of the YopM that was previously associated with thrombin, and the crosslinked complex of YopM and  $\alpha$ -thrombin is not obtained. YopM was in fact previously associated with thrombin, because the degradation product (dotted arrow) that was produced during the preincubation of thrombin and YopM is seen. Hirugen, the sulfated tail of hirudin which binds to abeI of  $\alpha$ -thrombin, also displaces YopM (not illustrated). These findings indicate that YopM binds to abeI.

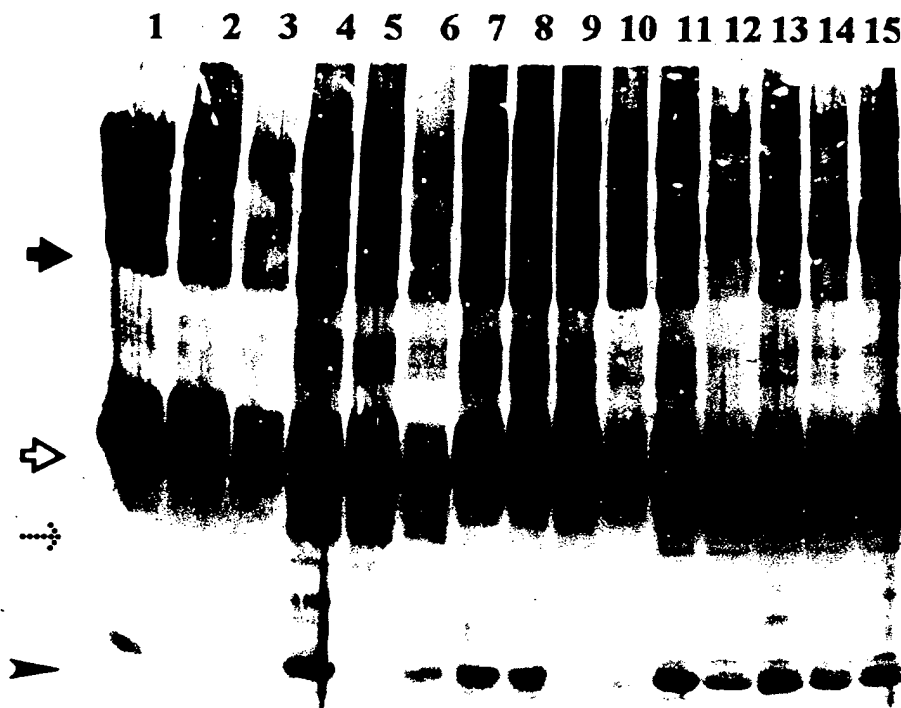


Figure 8. Crosslinking of YopM to  $\alpha$ -thrombin (lanes 1-6) or blocked  $\alpha$ -thrombin (lanes 7-15) with and without preincubation, and competition tests with hirudin and with DNA aptamer. Arrowhead symbols as in Figure 7. Concentrations of YopM and thrombin, respectively, were : 5  $\mu$ M and 5  $\mu$ M, lanes 1,4,7,8, and 11-15; 5  $\mu$ M and 0.8  $\mu$ M, lanes 2,5, and 9; 0.8  $\mu$ M and 5  $\mu$ M, lanes 3,6, and 10. Hirudin was used at 10 and 50  $\mu$ M (lanes 11 and 12, respectively). DNA aptamer was present at 200, 400, and 600  $\mu$ M (lanes 13,14, and 15, respectively).

Figure 8 proves that YopM is cleaved by thrombin.

a.) Negligible product (dotted arrows) is seen when YopM was mixed with thrombin and immediately crosslinked without any preincubation (lanes 1-3).

b.) The cleavage product is seen when YopM and thrombin are preincubated prior to crosslinking (lanes 4-6) (further degradation occurs if YopM is incubated with thrombin longer than we typically did in our crosslinking studies -- not illustrated). The cleavage product is not seen when hirudin or hirugen is present, regardless of whether the mixture is preincubated prior to crosslinking or not (not illustrated).

c.) YopM crosslinks well to thrombin in which the catalytic site is stably blocked with Phe-Pro-Arg-chloromethyl ketone (FPRck) (from Haematologic Technologies, Inc.) (lanes 7-10). However, the cleavage product is not seen with blocked thrombin, whether YopM is preincubated (lane 7) or not (lanes 8-10) with blocked thrombin before the crosslinking. These findings indicate that YopM binds weakly at thrombin's catalytic site, when thrombin is not blocked.

Figure 8, lanes 11 and 12 show that hirudin still can compete with YopM for binding to blocked thrombin, although higher concentrations are required than were for non-blocked thrombin (consistent with the known lower affinity of hirudin for blocked than nonblocked thrombin (20). In these samples, blocked thrombin and hirudin were preincubated before being mixed with YopM and immediately crosslinked. In lane 11, 10  $\mu$ M hirudin was used, and little competition occurred, whereas 50  $\mu$ M essentially abolished the crosslinking of YopM to blocked thrombin. Hirugen also will compete with YopM for binding to blocked thrombin (not illustrated). As hirudin and hirugen both bind to blocked thrombin at abeI, these findings indicate again that YopM binds to thrombin at abeI.

There is a DNA 15-mer (5'-GGTTGGTGTGGTTGG-3') that folds in such a way as to bind very tightly to abeI (21,22), and this aptamer inhibits chemical crosslinking of YopM to blocked thrombin (lanes 13-15). Blocked thrombin was preincubated with 200, 400, and 600  $\mu$ M aptamer in the samples of lanes 13, 14, and 15, respectively, before being mixed with YopM and crosslinked. These concentrations provided a 40- 80- and 120-fold excess of competitor (aptamer) compared to the concentrations of YopM and thrombin. The higher concentrations of aptamer significantly inhibited the formation of the complex of YopM and blocked thrombin (lanes 14 and 15). Hirudin or aptamer bind blocked thrombin at abeI (13-15); their inhibition of YopM's binding to blocked thrombin shows that YopM binds to blocked thrombin via abeI.

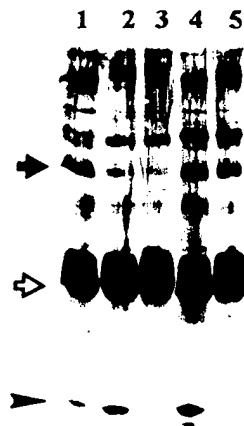


Figure 9. The competition effect of aptamer at *abeI* requires an aptamer structure that allows interaction of aptamer with *abeI*. Lane 1, YopM (5  $\mu$ M) and  $\alpha$ -thrombin (5  $\mu$ M) were mixed and immediately crosslinked; lane 2, 5  $\mu$ M  $\alpha$ -thrombin was preincubated with 100  $\mu$ M aptamer, then 5  $\mu$ M YopM was added and the mixture was crosslinked; lane 3, as in lane 2 except that 0.08  $\mu$ M  $\alpha$ -thrombin was used; lane 4, as in lane 2 except that scrambled aptamer was used; lane 5, as in lane 3 except that scrambled aptamer was used. Symbols as in Fig. 7.

Figure 9 shows that it is aptamer's specific structure, as opposed to its overall composition, that is important for its successful competition with YopM for thrombin. A 20-fold molar excess of aptamer over YopM prevents YopM from being crosslinked to thrombin (lane 2 compared to lane 1). If the thrombin concentration is reduced ca. 6-fold, which still allows strong formation of a crosslinked product (Figure 8 lane 2), a 5-fold molar excess of aptamer over YopM has a greater inhibitory effect (Fig. 9 lane 3). However, a scrambled aptamer with the same composition but different sequence (5'-GGTGGTGGTTGTGGT-3') does not prevent YopM from crosslinking to thrombin, even when the concentration of thrombin is decreased 6-fold (Fig. 9, lanes 4 and 5). This scrambled aptamer also is unable to bind at *abeI* (22). This indicates that aptamer likely has its effect by folding into a structure that can bind to *abeI* tightly enough to exclude YopM, rather than by nonspecifically binding to YopM or to thrombin. (The differences in amounts of aptamer needed for successful competition in Figures 8 and 9 [ $> 200 \mu$ M vs. 100  $\mu$ M, respectively] are due to the use of blocked thrombin in Fig. 8 and non-blocked thrombin in Fig. 9 [YopM appears to bind more strongly to blocked than non-blocked thrombin: Fig. 8, lane 7 compared to lane 1; lanes 8-10 compared to lanes 4-6]).



In summary, these experiments showed that YopM binds to thrombin at abeI and that this interaction is essential for YopM to be crosslinked chemically to thrombin. If abeI is blocked by a more tightly binding molecule, YopM does not bind stably to thrombin, and we do not obtain the YopM-thrombin crosslinked product; nor do we see YopM cleavage by thrombin. It is significant that YopM's interaction with thrombin was inhibited with relatively small molecules: the decapeptide hirugen and the DNA aptamer occupy only abeI when they bind to thrombin. There should be a minimum of nonspecific steric effects from such small molecules. This suggests that it is truly abeI or a region very near to it where YopM binds. YopM also can interact with thrombin's catalytic site, because YopM is cleaved by thrombin. However, this interaction does not constitute an essential part of YopM's affinity for thrombin, because YopM binds at least as well, if not more strongly, to blocked thrombin than it does to thrombin.

#### Where on YopM does thrombin bind?

Based on our findings, we need to look for regions of YopM that can form extended structures like those in hirudin or thrombin receptor, where there are electrostatic complementarity with the positive field produced by abeI and an appropriate sprinkling of hydrophobic residues to anchor the molecules together. The fact that there is no set consensus sequence but rather the more general "electrostatic complementarity" plus variable hydrophobic interactions confounds the straightforward identification of YopM's potential abeI-binding site by inspection of the predicted amino acid sequence of YopM.

YopM is a very acidic 367-residue protein (predicted pI = 4.06). It is not hard to find hirudin-like sequences; 7 of these are boxed in the sequence of YopM shown in Fig. 10.

```

      M F I N P R N V S N T F L
Q E P L R H S S N L T E M P V E A E N V K S K T E Y Y N A
W S E W E R N A P P G N G E Q R E M A V S R L R D C L D R

Q A H E L E L N N L G L S S L P E L P P
H L E S L V A S C N S L T E L P E L P Q
S L K S L L V D N N N L K A L S D L P P
L L E Y L G V S N N Q L E E L P E L Q N L P
F L T A I Y A D N N S L K K L P D L P L
S L E S I V A G N N I L E E L P E L Q N L P
F L T T I Y A D N N L L K T L P D L P P
S L E A L N V R D N Y L T D L P E L P Q
S L T F L D V S E N I F S G L S E L P P
N L Y Y L N A S S N E I R S L C D L P P
S L E E L N V S N N K L I E L P A L P P
R L E R L I A S F N H L A E V P E L P Q
N L K Q L H V E Y N P L R E F P D I P E

S V E D L R M N S E R V V D P Y E F A H E T T D K L E D D
V F E

```

Figure 10. Sequence of YopM. Boxed regions are sequences that resemble the *abeI*-binding C-terminus of hirudin.

However, a synthetic 13-mer peptide containing one of the hirudin-like sites that is exactly repeated in the YopM sequence (LEELPELQNLPPF) plus an N-terminal nonencoded C does not inhibit the YopM-thrombin interaction as measured by the competition crosslinking assay described above. The C-terminal 31 residues of YopM also present a highly negatively charged region. A synthetic 13-mer peptide containing the C-terminal 12 residues plus an N-terminal nonencoded C also did not interfere with YopM's thrombin-binding in the crosslinking assay. These negative results prompted further considerations of YopM's likely structure before selecting target regions for site-directed mutagenesis.

YopM is a member of the LRR protein family. YopM belongs to a family of proteins that contain a leucine-rich repeat (LRR) motif (23). In porcine ribonuclease inhibitor (RI), the prototype LRR protein for which X-ray data are available, these tandem repeats create an extended structure of parallel  $\beta$ -strands connected by  $\alpha$ -helices. The definition of the LRR consensus for the LRR family includes the  $\beta$ -sheet feature of the RI structure, which is stabilized by L residues and a conserved N. However, the residues between these Ls and N are variable. The connections between the  $\beta$  strands in the LRR family members are not necessarily  $\alpha$ -helices as in RI; in YopM, at least some of these connections are likely to be short kinky loops. YopM was

predicted by Kobe and Deisenhofer to have 12 tandem repeats (23). We arranged YopM's sequence in Fig. 11 to show a potential 13th repeat at the beginning of the stretch of repeats. This one, however, has the conserved N shifted by one residue.

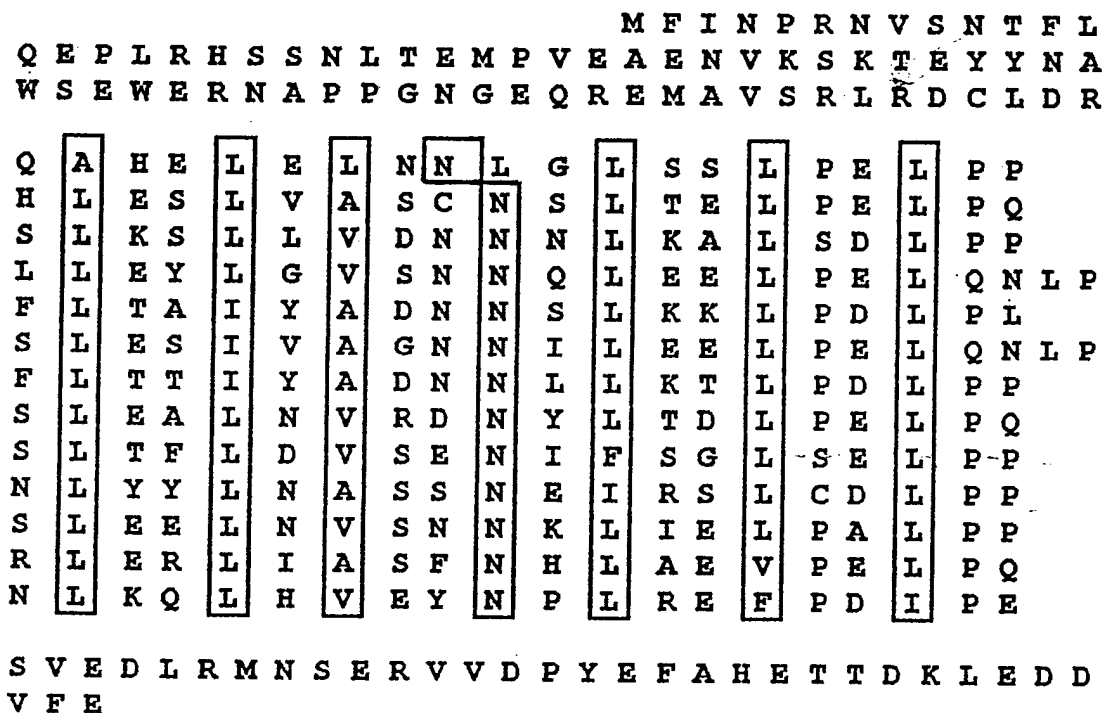


Figure 11. YopM sequence, with LRR conserved residues boxed. Residues 72-335 are arranged in 13 rows, where each row is a LRR.

Although one other LRR family member binds thrombin (the  $\alpha$  chain of platelet glycoprotein GPIb), no other LRR protein binds thrombin, and GPIb $\alpha$  is thought to bind thrombin in a "hinge" region that lies C-terminal to the LRR cluster. The commonality among LRR proteins boils down to the vague summary that all of these proteins interact with other proteins, many functioning at the cell surface.

Molecular modeling of YopM; X-ray structure data for YopM. So how would a LRR protein bind thrombin at *abeI*? Modeling YopM's likely structure based on its LRR nature might help us identify hirudin-like regions that are accessible and flexible enough for the interaction with thrombin. We hope to apply molecular modeling algorithms to YopM as one means of identifying likely *abeI* binding sites for us to mutagenize. We also have sent 20 mg YopM (not cleaned of endotoxin) to Brian Edwards at Wayne State University, who is attempting to crystallize it. If that effort is successful, a bonanza of information will be gained about YopM's structure and its potential mechanism of binding  $\alpha$ -thrombin.

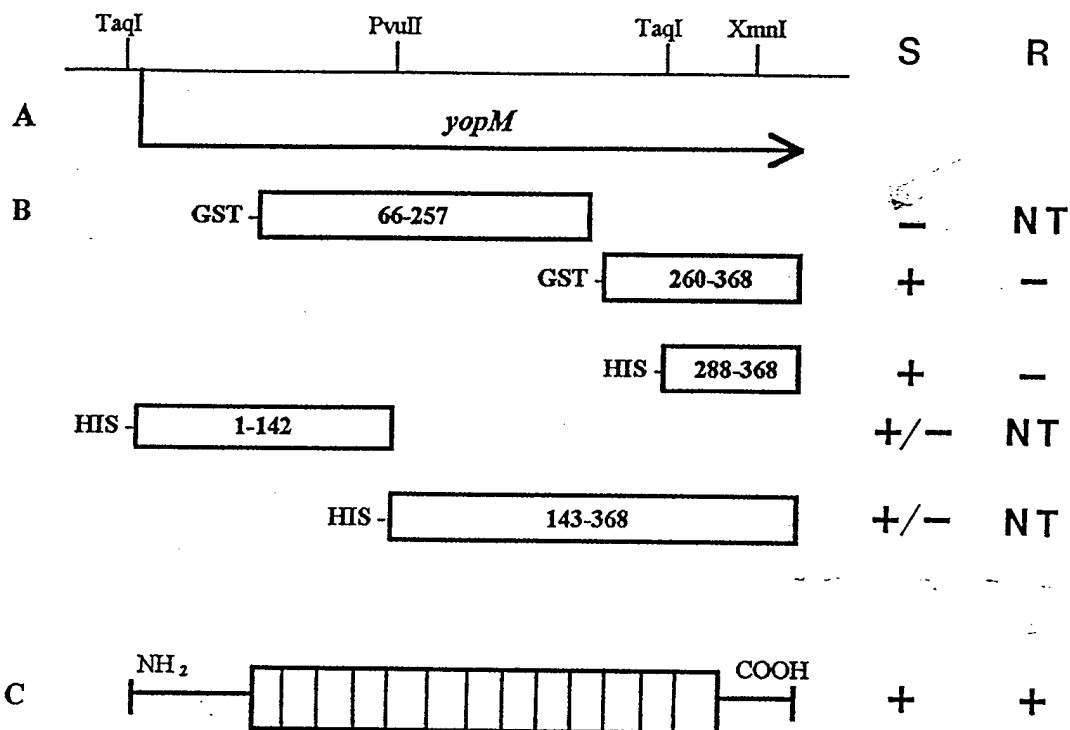
Rough localization of the region of YopM that binds to thrombin. As a third simultaneous approach, we are testing portions of YopM for their ability either to bind to thrombin or competitively inhibit YopM's thrombin-binding. We took the approach of creating fragments of YopM by molecular genetic methodology. The overall strategy is to place the coding region of a portion of YopM in frame with an upstream sequence coding for six histidines (His-Tag fusion protein method; pPROEX1 vector (Promega) and affinity resin (sold by Qiagen) or for the enzyme glutathione-S-transferase (GST; pGEX-3X vector and affinity resin sold by Pharmacia). The resulting fusion proteins are expressed in *Escherichia coli* and purified on Ni-NTA or glutathione affinity resins, respectively. This approach potentially permits the synthesis of any YopM fragment and provides a simple method for purification of the fragment. If desired, the N-terminal H<sub>6</sub> or GST can be cleaved and removed from the fusion protein, although full function is often shown by intact fusion proteins. The negative features of this approach are that the resulting YopM fragment might be insoluble (a pitfall also of the alternative chemical or proteolytic cleavage methods) or that the fusion construct might be poorly expressed in *E. coli*. We have encountered both of these problems in the course of our work. It also is possible that portions of proteins might not maintain their normal configuration in solution, a pitfall inherent in any method that uses peptides or fragments of proteins, regardless of how these are created. All of these potential pitfalls make it imperative to have more than one independent approach for analyzing where YopM's thrombin-binding site is.

Creation of fusion constructs. PCR was used to synthesize the desired yopM fragments, using the YopM-containing clone PBS10 (3) as template. GST-YopM 66-257. For the PCR, the upstream primer was 5'-TCGTGGGATCCGAGATTGCCTGGACCGA-3' and the downstream primer was 5'-ACGATGAATTCAAGTTTGGTGGCAATTC-3'. The upstream primer contained a *Bam*HI site, and the downstream primer contained an *Eco*RI site, at their 5' ends. The PCR reactions and subsequent DNA manipulations were carried out as previously described (24), except that the 25 to 30 cycles of amplification employed 1 sec for each step (94, 55, 72°C) in a Perkin Elmer GeneAmp model 2400 thermocycler (Perkin Elmer Cetus). The PCR fragment was digested with *Bam*HI and *Eco*RI, and the 596 basepair (bp) product was cloned into *Bam*HI/*Eco*RI-digested pGEX-3X. This and all other constructs described below were sequenced to verify that the fusion junction was correct and that no errors had been introduced by the Taq polymerase (Promega). The resulting fusion protein migrated at ca. 50-55 kDa in SDS-PAGE. GST-YopM 260-368. This construct was made as described above, with upstream primer 5'-TCGTGGGATCCTCAATGCATCCAGCAAT-3' and downstream primer 5'-ACGATGAATTCTACTCAAATACATCATC-3'. The PCR product was 346 bp, and the resulting fusion protein was ca. 35-40 kDa in mass. HIS-YopM 288-368. The PCR product GST-yopM 260-368 was digested with TaqI (see Fig. 12) and *Eco*RI and cloned into *Nar*I/*Eco*RI-digested pPROEX1. The resulting 102 amino-acid fusion protein was ca. 11

kDa in mass. HIS-YopM 1-142. The PCR utilized upstream primer 5'-ATGTACTAGGGCGCCATGTTTCATAAATCCAAGAAATGTATCT-3' and downstream primer 5'-TTCGACTTCCAGCTGATTATTAGAGATGACACCTAAATATTCCAG-3', which, respectively, contained a *NarI* or *PvuII* site at their 5' ends. The PCR product was digested with *NarI* and *PvuII*, and the 426 bp fragment was cloned into pPROEX1 digested with *NarI* and *StuI*. The resulting 163 amino-acid fusion protein was ca. 18 kDa in mass. HIS-YopM 143-368. The PCR utilized upstream primer 5'-CCACCTTTACTGGAATATTTAGGTGTCTCT-3' and downstream primer 5'-ACGATGAATTCTACTCAAATACATCATC-3', which, respectively, contained a *PvuII* or *EcoRI* site at the 5' end. The PCR product was cleaved with *PvuII* and *EcoRI*, and the 682 bp fragment was cloned into *EheI/EcoRI*-digested pPROEX1. The resulting 248 amino-acid fusion protein was ca. 27 kDa in mass.

Production of fusion proteins. *E. coli* containing the fusion construct or the vector alone (as a control) were grown in Luria Broth (LB), expression of the fusion construct was induced by addition of IPTG, the bacteria were broken by passage through a French pressure cell, and the lysate was separated into soluble and insoluble fractions, as previously described (25). The fusion protein was purified by affinity chromatography, proteolytically cleaved of its fusion partner, and purified from its fusion partner, as described by the supplier of the affinity resin and protease (Promega and Pharmacia for His-Tag- and GST-related materials, respectively). For the GST fusions, *E. coli* DH5 $\alpha$  (Gibco-BRL) was used for cloning and expression. For the His-Tag fusions, the *dam E. coli* GM2163 (26) was used. Slightly better expression of HIS-YopM 1-142 and HIS-YopM 143-367 was obtained in the *ompT E. coli* BL26 (Novagen) than in *E. coli* GM2163. Growth was in LB at 37°C, unless expression was poor or inclusion bodies were formed, in which case we tested for improvement of the yield of soluble fusion protein after growth at lower temperatures (30 or 26°C) (27) and in a richer medium (28), as noted below.

Figure 12 summarizes the constructs made and indicates whether they are soluble ("S") and whether they are reactive ("R") -- i.e., whether they successfully inhibit YopM's thrombin binding in the competitive crosslinking assay.



**Figure 12. *yopM* fusion constructs and the characteristics of their encoded product.** Panel A shows the *yopM* gene, with several restriction sites indicated. Panel C diagrams the YopM protein, with the boxes representing the 13 LRRs. Panel B diagrams 5 fusion constructs, showing the portions of the YopM gene they contain and the region of the YopM protein that they encode. The residues of YopM specified by the constructs are indicated by the numbers. ("Residue" 368 is the stop codon.) HIS designates an N-terminal H<sub>6</sub>, and GST designates an N-terminal GST enzyme, in the fusion protein. For each construct, + or - indicates the solubility (S column) of the fusion protein, or the reactivity (R) of the fusion protein in the competitive crosslinking assay. NT = not tested.

YopM is predicted to be more hydrophilic at its N-terminal and C-terminal ends than in the central portion (7), which may explain why GST-YopM 66-257 formed an inclusion body, whether the bacteria were grown at 37°C or 30°C. This inclusion body did not solubilize significantly upon treatment with 5M urea or with a dialyzable detergent such as 1% Triton-X-100, and we were not able to obtain material to test in the crosslinking assay. In contrast, the two fusion proteins that contained YopM's C-terminus, GST-YopM 260-367 and HIS-YopM 288-367, were soluble and cleavable with Factor Xa and TEV protease, respectively. However, in one test made, they did not inhibit YopM's binding to thrombin in the competitive crosslinking assay. We will repeat

this test, as we had made only a limited amount of fusion protein and had only estimates for the amounts added to the assay. If our negative competition result is reproducible, then we hypothesize that the C-terminal-most two hirudin-like sequences in YopM (Fig. 10) are not directly involved in binding to thrombin.

We hoped that by making HIS-YopM 1-142 and HIS-YopM 143-367 we could divide the YopM molecule ca. in half and retain solubility by having the hydrophilic N- or C-terminus present. This would permit us to assign thrombin-binding to the N- or C-terminal half of the protein. These constructs have given problems with both low-level expression and insolubility of the product. Expression of both of these fusions is better at 37°C than 26°C and in *E. coli* BL26 than GM2163. Expression was better in rich medium (28) than in Heart Infusion Broth (Difco) than in LB. Even under the best conditions, the fusions are so poorly expressed that they can't be seen in Coomassie-stained SDS-PAGE gels of the bacterial fractions (we detected them by immunoblot). Moreover, most of the fusion protein that was expressed was present in inclusion bodies. However, these appear to be soluble in 5M urea, opening the possibility that we may be able to renature them by dialysis against stepwise decreased concentrations of urea. We may be able to obtain enough protein for use in crosslinking or competitive crosslinking assays by processing a large volume of culture and diluting all reactants in the assay. We already know that YopM can be crosslinked to thrombin at 20 nM concentrations of reactants (ref. 3; detection was by autoradiography for the emission from <sup>125</sup>I-labeled YopM). We could enhance our detection capabilities in immunoblots, e.g., by using <sup>125</sup>I-labeled secondary antibodies to analyze crosslinking or competitive crosslinking assays.

If we are unable to obtain information from HIS-YopM 1-142 and 143-368, we will discontinue efforts to make fusion proteins containing the central, LRR-containing part of the YopM molecule. It could be that this is the part of YopM that contains the putative hydrophobic patches manifested by strong adherence of YopM in hydrophobic interaction chromatography and by oligomerization of YopM during gel filtration or at pH > 8. The LRR part of YopM may require the rest of YopM for proper folding and for control over YopM's stickiness.

In summary, the fusion protein approach so far has indicated that the C-terminus of YopM is not directly involved in binding to thrombin. The caveats with this conclusion are: 1) we need to be sure that we have tested a sufficiently high concentration before we conclude that a C-terminal fragment fails to inhibit YopM's thrombin-binding and 2) we cannot know whether the LRRs in a fragment containing only 2 or 3 LRRs are able to stably form their native structure (so that failure of competition by the fragment might not rule out a role in thrombin-binding for the intact structure). Consistent with the findings from fusion proteins, the test for inhibition of YopM's thrombin-binding by the very C-terminal 12 residues indicated that these residues are

not sufficient to inhibit YopM's thrombin binding.

Information from crosslinking experiments. We have 2 additional pieces of information about where thrombin binds on YopM.

1) The product of cleavage of YopM by thrombin is ca. 3 kDa lower in mass than is YopM and is missing its C-terminus, judging by our inability to detect it with our anti-peptide antibody raised against the last 12 residues of YopM. This suggests that YopM is processed at the C-terminus and therefore that a C-terminal R can interact with thrombin's catalytic site. On thrombin's substrates, the abeI-binding site can be as far as 51 residues away from the point of cleavage (for the B $\beta$  chain of fibrinogen [16]). Hence, if YopM binds thrombin as does fibrinogen, then its abeI binding site ought to be in the C-terminal quarter of the molecule.

2) The crosslinked product of YopM and thrombin (or blocked thrombin) was obtained with the heterobifunctional crosslinker SMCC (which targets SH/NH<sub>2</sub>), indicating that a cysteine on either YopM (which has 3 C) or thrombin (which has 8 C) is close to a region where the two proteins interact. As none of thrombin's Cs is a free -SH group (19), this experiment told us something about where on YopM thrombin binds: one of YopM's 3 Cs is close to a surface-exposed residue on thrombin that reacts with the N-hydroxysuccinimide ester of SMCC. As  $\epsilon$ -amino groups react by far the best with this ester, our experiment suggests the hypothesis that a surface-exposed K on thrombin is close to a C on YopM. YopM's 3 Cs are residues 68, very near to the start of the first LRR; 100, in the 2<sup>nd</sup> LRR; and 271, in the 10<sup>th</sup> LRR. This last C lies within the fragment of YopM containing residues 260-367, which did not inhibit YopM's thrombin-binding in the competitive crosslinking assay (Fig.12) (but recall that we need to repeat that test with known amounts of competitor protein fragment). The regions surrounding YopM's first 2 Cs have not yet been tested in competitive crosslinking assays.

We believe that it is worthwhile to think in terms of a linear sequence in YopM rather than an assembled topological structure that binds to abeI, because so far, all of thrombin's many substrates and natural inhibitors bind to abeI by extended linear structures. Our studies have examined the possible importance of 4 of the 7 sites boxed in Fig. 10: two in the middle of the molecule are tentatively ruled out by the failure of the peptide containing their sequence to inhibit YopM's binding to thrombin; we need to confirm whether the C-terminal two are not involved in thrombin-binding by retesting for the ability of the C-terminal fragments from YopM GST-YopM 260-367 and HIS-YopM 288-367 to inhibit YopM's thrombin-binding. We will



direct our next tests toward determining if the N-terminal part of YopM interacts with *abeI*. This region might not be locked up in LRR-associated 3° structure and might be available to bind thrombin as a linear sequence. Then we will begin higher-resolution probing and site-directed mutagenesis to create a YopM that is defective in binding to thrombin (Aim 2, TO 2).

Aim 3. Determine the fate of YopM when *Y. pestis* interacts with phagocytic cells.

This part of the project has not started yet. We are making two reagents that we will need for this study: rabbit antibody directed against YopM (as the antibody reagent made in mice will be limited in amount) and rabbit antibody directed against a control protein whose fate is known. We will use YopE for this latter purpose (1,29). These antibodies are already being raised during the course of non-DOD-supported research in my lab, and the anti-YopM antibody is now ready. This USAMRDC-supported project contributed the YopM for immunization and a small part of the time required by personnel to harvest and purify the antibody.

It should be noted that the personnel requested for this part of the project was cut from the budget. Hence, this part of the work needs to draw effort from the two budgeted people who have devoted most of their effort on Aims 1 and 2, which received highest priority by DOD. As a result, we expect this part of the project to proceed more slowly than planned in the original proposal.

Conclusions

Aim 1.

1. We have purified > 1g YopM and have demonstrated that it is free of contaminating *Yersinia* proteins according to the criteria we originally set forth in the proposal. Although the YopM we have on hand is satisfactory for the proposed studies of Aim 1, we may find ourselves limited for un-nicked YopM for the structural/functional studies of Aim 2, and we will test whether a different expression construct produces a greater fraction of YopM in an un-nicked state.

2. We learned that YopM can oligomerize as multiples up to hexamers and that this is promoted by  $\text{pH} \geq 8.0$  and by interaction of YopM with agarose-based resins used for gel filtration chromatography. This property may be indicative of a YopM activity not previously suspected.

3. We are able to remove endotoxin from YopM to a level that satisfies our criteria of < 1 ng/dose/mouse, but this process is expensive, and we will make some tests to see if we can find an alternative, less expensive method. However, we will devote only

the time and cost needed to clean up enough YopM for our proposed studies and for the 5 mg of YopM that we will provide as a deliverable to USAMRIID.

4. We have begun the process of collecting anti-YopM antibody and control antibody in mice. We are disappointed in the titers that we are obtaining in the ascites and need to use more mice than originally projected. For these extra mice, we plan to obtain antibody from serum rather than from ascites. Even so, this reagent will be a precious commodity, and we will carefully focus our work so as to achieve the scientific goals within the limits of availability of these antibody reagents.

5. We developed a YopM ELISA and a YopM competition ELISA for analyzing the antibody response to YopM.

6. We tested two adjuvants and found that Freund's adjuvant (FA) elicited anti-YopM antibody more quickly in mice than did Adjuvax. However, mice treated with Adjuvax eventually produced titers comparable to those in mice that received FA. We will use FA in our remaining studies, because we need to be as expeditious as possible to complete the proposed studies within the 3-year project period. Moreover, in our mouse model, we are using intraperitoneal (IP) immunization, where the FA merely promotes the formation of relatively innocuous granulomas within the body cavity rather than the necrotic surface lesions that would be obtained with the use of FA for peripheral routes of immunization. Hence, the use of FA in this model should not raise animal welfare concerns.

7. We found that the predominant antibody isotype elicited by immunization of mice with YopM is IgG1, regardless of the route of immunization, where we tested IP, subcutaneous, intramuscular, and intradermal routes. We will use IP for the remainder of our work, because it is the most effective and most appropriate for our experimental systemic plague model.

8. We have begun the active immunization of mice with YopM to test directly if YopM is a protective antigen.

#### Aim 2.

9. We modified the chemical crosslinking assay into a competition format to allow us to determine where on thrombin YopM binds.

10. Using this assay, we determined that YopM binds to thrombin's anion binding exosite I (abeI), which is one of two exosites at which thrombin's natural substrates and inhibitors bind. This finding focuses our efforts to identify where on YopM thrombin binds: we need to identify on YopM an accessible linear sequence containing 1) several negatively charged residues to counter the positive electrostatic charge of abeI and 2) a sprinkling of

hydrophobic residues to insert into the hydrophobic crevices of abeI.

11. We also found that YopM is cleaved by thrombin. This indicates that YopM can associate with thrombin's catalytic site, although this association is not essential for YopM's binding to thrombin, because YopM seems to bind best to thrombin that has a blocked catalytic site. As the cleavage product lacks YopM's C-terminus, YopM must be processed at its C-terminus by thrombin. This suggests that the C-terminal part of YopM must be able to interact with thrombin, at least at thrombin's catalytic site.

12. Recent improved understanding of the structure of the leucine-rich repeat (LRR) regions of the LRR family of proteins has aided our visualization of YopM's likely structure and thus helps us to interpret our structure/function findings and to focus our efforts toward identifying where on YopM thrombin binds.

13. We plan to use available structural information about the LRR repeat component and about how thrombin binds to its substrates to analyze YopM further by molecular modeling. This can help us learn how YopM might bind thrombin and can guide our choice of residues on YopM to be altered by site-directed mutagenesis.

14. We are supplying pure YopM not cleaned of endotoxin to Brian Edwards of Wayne State University, who is attempting to crystallize YopM. This may not be successful; but if it is, we will gain a wealth of information about YopM that will help determine how YopM binds thrombin, how it may oligomerize or potentially interact with other proteins, and how future efforts might best create a YopM toxoid if that is needed prior to using YopM as a vaccine for US troops.

15. Using PCR, we made 5 genetic constructs for the production of fragments of YopM to be used in the competitive crosslinking assay to determine roughly where on YopM thrombin binds. One of these expresses an insoluble product, and its further use has been discontinued. Two expressed products are of limited solubility and amount, but we may be able to use them in a modified assay, if we are able to obtain enough soluble form. Two constructs made soluble products which we were able to test for their ability to inhibit YopM's thrombin-binding. They failed to inhibit, suggesting that the C-terminal ca. 30% of the YopM molecule does not contain an intact thrombin-binding site. We will repeat those tests with known amounts of competitor protein.

16. A heterobifunctional crosslinker that targets an SH and an NH<sub>2</sub> group crosslinks YopM to thrombin. As all of thrombin's cysteines are tied up in disulfide linkages, this indicates that one of YopM's 3 Cs is near enough to where YopM binds thrombin to

be crosslinked. We already may have ruled out one of these Cs by the failure of a fragment containing it to inhibit YopM's thrombin-binding. We will confirm that result and then will focus our attention to the N-terminal part of the YopM molecule, which contains the other 2 Cs.

17. After roughly localizing YopM's thrombin-binding site, we will begin higher-resolution localization of likely residues involved in the interaction. We hope to begin making and characterizing mutant YopMs before the second year of funding ends.

### Aim 3.

18. This part of the project has not been formally begun, although we have generated one of two antibody reagents we will need. We are currently generating the other. It should be noted that the personnel requested for this part of the project was cut from the budget. Hence, this part of the work needs to draw effort from the two budgeted people who have devoted most of their effort on Aims 1 and 2, which received highest priority by DOD. As a result, we expect this part of the project to proceed more slowly than planned when the proposal was written.

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